The dosage optimization of He-Ne laser energy as a candidate for photodynamic therapy of cancer cells with exogenous photosensitizer variations

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The dosage optimization of He-Ne laser energy as a candidate for photodynamic therapy of cancer cells with exogenous photosensitizer variations

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Abstract. The aim of this research was to find out the most effective energy dose of laser energy per unit area irradiated to the subject, used in T47D cancer cell photodynamic therapy. The experiment used three variations of energy doses and two exogenous photosensitizers. The laser used was the He-Ne laser with a wavelength of 632 nm. Laser exposure on the T47D cancer cells was conducted in vitro at a distance of 1 cm with energy doses of 30.4 J/cm^2, 18.8 J/cm^2, and 30.4 J/cm^2. The photosensitizers used in the experiment were chlorophylls isolated from sour-sop leaves and *Protoporphyrin IX* (*PpIX*). The concentration of photosensitizers which is not toxic to cells, based on the toxicity test, is 300 ppm for chlorophylls and 350 ppm for *PpIX*. At the energy dose of 30.4 J/cm^2, the percentage of cell death was 96.25% with chlorophyll addition, while with *PpIX* addition, the percentage was 81.62%. At the same energy dose, cell death in apoptosis was 95.78% with chlorophyll addition and 80.56% with *PpIX* addition. Based on the result of this research, it was revealed that the energy dose of He-Ne laser output of 30.4 J/cm^2 was the most effective energy dose between the energy dose variation that could yield T47D cell death in an apoptosis way.

1. Introduction

Cancer is a disease caused by abnormal and uncontrollable body tissue's cell growth. The prevalence of cancer in Indonesia is 1.4 per 1000 people and has become a major cause of death around the world [1]. At the moment, there are several alternative medications for cancer with their own downsides. As a consequence, alternative medication which is minimally invasive and selective against cancer cells is needed.

One type of medication that can be an alternative against cancer is photodynamic therapy [2]. Photodynamic therapy involves three main components: light, photosensitizer, and oxygen.
Chlorophylls and Protoporphyrin IX (PpIX) have chromophore and auxochrome clusters, allowing them to be made as photosensitizers. Both of them have absorption spectrums within the red

wavelength [2]. In photodynamic therapy, there is a process where photosensitizers are localized on cancer tissues and when exposed with lasers the photosensitizers become active and produce Reactive Oxygen Species (ROS). ROS then induces apoptosis death. Apoptosis is an organized and programmable death, so pathologically only apoptosis death is allowed [3].

Li *et al.* (2010) have stated that the combination of C-phycocyanin and He-Ne laser irradiation is able to increase the activity effects of anti-tumor, body immune, and pro-apoptosis against cancer cells [4]. Similar research was also conducted by Al-Khafaji *et al.* (2010) who showed that the most damaging effect on cancer cells resulted from the combination of Hematoporphyrin derivative photosensitizers and He-Ne laser irradiation on low power with energy doses of 3.6 and 7.2 J/cm^2 [5]. Fadilla (2015) also conducted research using PpIX and chlorophylls with radiation from lasers with a wavelength of 650 nm, and a laser energy dose of 18.8 J/cm^2 could cause apoptosis death to cancer cells [6]. The weakness of this research is that the laser interaction effects on cells could not be achieved with an effective energy dose. Therefore, the aim of this research was to find out the most effective energy dose of lasers using non-toxic photosensitizer solutions between variation of energy doses similar to the previous studies.

2. Research Methods

The laser used in this research is the He-Ne laser LSW-10 from Jianxi Liansheng Technology Co., Ltd with a wavelength of 632 nm. The stability of the laser was characterized using an experimental setup shown in Figure 1.

Chlorophylls as photosensitizers in this research were extracted from the leaves of sour-sop. Sour-sop leaves were chosen because they can grow abundantly only from one tree which will not disturb the ecosystem by endangering the existence of sour-sop plants due to exploitation. They were also chosen because they are not a food commodity so they can be used for medical purposes as photosensitizers to cure cancer due to their chlorophyll content. The chlorophyll isolation was conducted by drying the sour-sop leaves that had been cut into small pieces in the open air but without direct sunlight. The process was then continued with maceration, evaporation, partition, and VLC (Vacuum Liquid Chromatography) to obtain 11 fractions of sour-sop leaf extract as shown in Figure 3. The green bluish fraction is the chlorophylls [8]. Then, a thin layer chromatography was conducted and the fractions were photographed with 366 nm UV light to know the fraction node patterns and to determine fractions with the most chlorophylls. The chosen fractions were then dried further in a 40°C oven to gain thick chlorophyll extracts as shown in Figure 4.

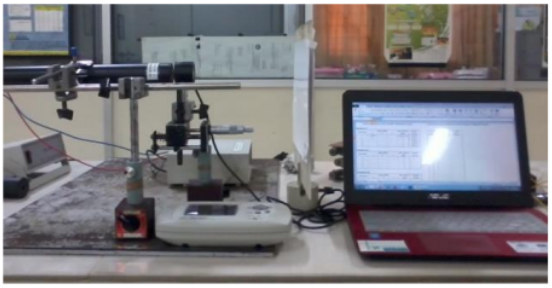


Figure 1. Checking of He-Ne Laser Power with A Digital Powermeter.

Another photosensitizer also used in this research was the commercial Protoporphyrin IX (PpIX) from Sigma-Aldrich. Absorption wavelength characterization was then conducted on both photosensitizers using a UV-Vis spectrophotometer.

Simultaneously, the T47D cancer cell culture reproduction in a petri dish using a set of cell culture tools such as a micropipette (Thermo), a centrifuge, a CO_2 incubator (Thermo), a 96-well plate, a conical tube (Biologix), a vortex, a light microscope (Nikon), ELISA Reader (Bio-Rad iMark), air flow laminar (Dalton), a fluorescence microscope (Olympus). The growth medium used in the culture reproduction of T47D cancer cells is DMEM medium with PBS (Phosphate Buffered Saline) 10%, Penicillin-streptomycin 1%, and fungison 0.5%.

Before the treatment to expose the laser to cancer cells with and without the photosensitizer, there was a photosensitizer toxicity test toward cancer cells with the MTT Assay method to find out the concentration of photosensitizers which was not toxic (safe) to cells. In the toxicity test, the photosensitizers (chlorophylls and PpIX) were prepared, with varied concentrations: 100 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm, and 350 ppm. The photosensitizers were solved with DMSO 0.1% solvent in DMEM cancer cell growth medium. DMSO 0.1 % was chosen because it was safe to cells [7]. A safe to cell photosensitizer solvent was chosen to avoid the influence on the result of the photosensitizer toxicity test.

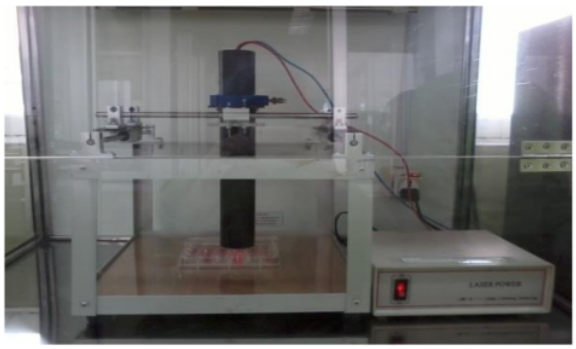


Figure 2. Setting Up the T47D Cancer Cell Exposure with Red He-Ne Laser inside Laminar Air Flow (LAF).

Figure 3. Eleven fractions produced from the VLC process.

Figure 4. Thick chlorophyll extract after being in an oven.

T47D cancer cells which had been grown to reach enough population for the test, were then divided into sample groups. There were three treatment groups, namely a laser without photosensitizer addition (L), a laser with chlorophyll addition (LK), and a laser with PpIX addition (LP). Cells were exposed with lasers inside Laminar Air Flow (LAF) so that they were still in sterile condition, with mountings and a slider that had been arranged in such a way that the lasers could slide along the X and Y axis (Figure 2). Before the exposure began, the laser mounting tools were cleaned first with alcohol.

Cells that had been incubated with chlorophyll and PpIX photosensitizers were put on a sample plate. Then, cells in each well were exposed with the He-Ne laser one by one with a variation of energy doses of 7.2 J/cm^2, 18.8 J/cm^2, and 30.4 J/cm^2. These energy doses are used due to an effective dose for the goals of the therapy. Each treatment was repeated three times. After the exposure finished, the cells were observed with a fluorescent microscope with an addition of coloring reagents, Acridine Orange (AO) and Ethidium Bromide (EB), to know the number of living and dead cells, based on the different colors produced. The living cells percentage was calculated using equation (1).

$$\%\ \text{living cells} = \frac{\text{OD Treatment} - \text{OD Media}}{\text{OD Cell Control} - \text{OD Media}} \times 100\% \quad (1)$$

3. Results and Discussion

3.1. Checking of the He-Ne laser power

Checking the power of the He-Ne laser was conducted by varying the laser distances from the samples, which were 1.0 cm up to 3.0 cm. Power measurements were conducted repeatedly up to three times, and an optimum average power of (0.603 ± 0.010) mW from the distance of 1 cm was obtained. Laser power changes due to variations of exposure distances were proven to be insignificant, which had a significant value of 0.128 .05), so the laser power was stated as stable against changes in exposure distances.

In checking the stability of the He-Ne laser power against the exposure time, it was revealed that the He-Ne laser in the distance of 1 cm started to be stable 30 minutes after turning on the laser. Besides power, the calculation of laser energy doses also considered the laser area that could be measured if the diameter was known. Laser has a Gaussian file in which the Gaussian file diameter depends on the laser exposure distance. In this research, the laser file diameter was adjusted with the data sheet of LSW-10 He-Ne laser which was 1.2 mm.

3.2. Photosensitizer characterization (chlorophyll and protoporphyrin IX)

Based on a photosensitizer absorbency test, it was as expected that chlorophyll had the highest absorbent peaks at the red and blue wavelengths (Figure 5), which was in line with the statement of Lichtenthaler and Claus in 2009 [9]. This result indicates that our isolation method is appropriate for this current work. As seen in Figure 6, in PpIX photosensitizer there were several absorbent peaks in the wavelengths of 462 nm, 542 nm, 598 nm, and 646 nm (red area wavelength). This is the same with research conducted by Rossetti *et al* in 2010 who found that the absorbent spectrum of PpIX showed a strong absorbency in the wavelength of 406 nm, and four weak peaks in the wavelengths of 516 nm, 554 nm, 600 nm, and 626 nm (red area wavelength) [10].The results of photosensitizer characterizations of both chlorophylls and PpIX were found to have the absorbency peaks in the red wavelength that were the same with the wavelength of the laser used in this research. The chlorophyll absorbency at the red wavelength (λ = 671 nm) is 0.129 while for the PpIX, the absorbency at the red wavelength (λ = 646 nm) is 0.088.

3.3. Photosensitizer toxicity test

The reading of toxicity tests was shown by Optical Density (OD) values which represent the number of living cells. Overall, with concentration variations from 100 ppm until 350 ppm, the toxicity tests showed that the percentage of living cells was more than 50%, so it could be concluded that with those concentrations, both photosensitizers (chlorophylls and PpIX) were not toxic. The concentrations of each photosensitizer with cell percentages are 300 ppm for the chlorophylls with the percentage of living cells of 97.6% and 350 ppm for PpIX with the percentage of living cells of 75.9% were chosen for the treatments with sample cells.

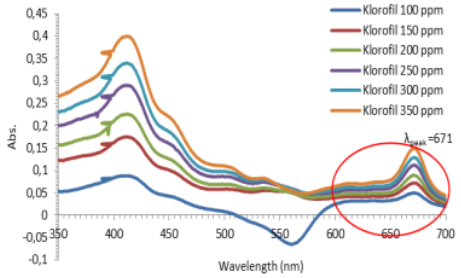


Figure 5. Chlorophyll Absorbance with Various Concentrations via UV-Vis Spectrophotometer.

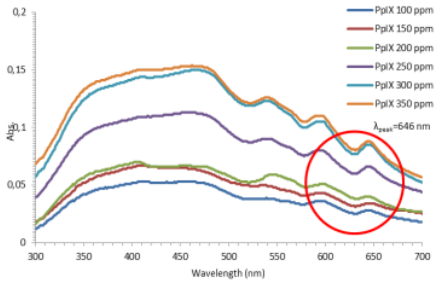


Figure 6. Absorbance of Protoporphyrin IX with Concentration Variations through UV-Vis Spectrophotometer.

3.4. T47D cancer cell death due to the red He-Ne laser exposure with or without photosensitizer additions

The percentages of cell death after the treatment in each group of L, LK, and LP with certain laser energy doses are presented in the form of a graph in Figure 7. As seen in the Figure 7, the highest cell death percentage occurred with the laser energy dose of 30.4 J/cm^2 and LK treatment was more effective in producing cell death compared to other groups of L and LP. It should be noted that the energy dose that became the independent variable in this research was the laser output energy dose (air dose), not the energy dose which was absorbed by the tissue of cancer cells (quantum yield).

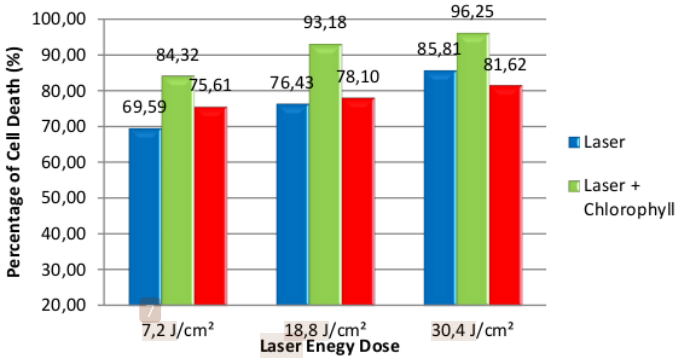
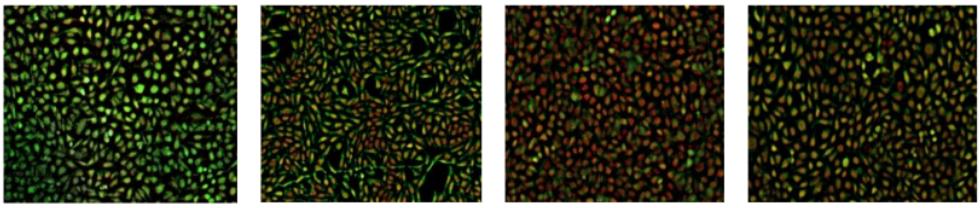


Figure 7. Graph of the Percentage of Cell Death After Treatment.

3.5. Observation of the number of apoptosis cells on T47D cancer cells after treatment

Coloring reagents (AO and EB) were given to differentiate the colors of the living cells and apoptosis ones. The difference in colors comes from the DNA fluorescent which is not same for each type of cells. Living cells with intact membranes have a uniform green color. When cells are in apoptosis stage and the membranes start to bleed, EB can enter into the cells and give an orange color. The picture of T47D cancer cells which was obtained from observation with a fluorescent microscope is divided into their treatment groups of control group, L, LK, and LP, respectively, as seen in Figure 8 and the percentage of apoptosis cell death due to treatments on each group is presented in the form of a graph as shown in Figure 9.

This graph shows that cancer cells exposed to the lasers with additions of photosensitizers with PpIX or chlorophylls were able to influence the cell death, and an addition of chlorophyll photosensitizer was found out to be more effective in affecting the cell death in an apoptosis way than addition of PpIX photosensitizer. This is because chlorophylls have more chromophore clusters than PpIX because chlorophylls experience more photophysical and photochemical interactions due to the more energy being absorbed from the given laser. Besides that, the peak absorption at the wavelength of 671 nm which falls into optical window range (650 nm – 850 nm) has quite high tissue penetration and enough triplet state energy to produce singlet oxygen. Meanwhile, PpIX has an absorption peak of a wavelength less than 650 nm (outside the optical window range for PDT, making it less effective in producing singlet oxygen). This is the reason why apoptosis cell death occurred more in the laser treatment group with chlorophyll addition (LK).



(a) (b) (c) (d)

Figure 8. T47D Cancer Cells after Treatment; (a) Control group (b) L (c) LK (d) LP.

This cell death caused by laser exposure with a photosensitizer addition is due to the interaction between the laser and the tissue. In the photodynamic therapy on the cancer cells, there is a photophysical and photochemical interaction mechanism whereas when photosensitizers are activated by light there is a production of Reactive Oxygen Species (ROS). ROS, especially singlet oxygen, can easily react with many biological substrates such as amino acids, various unsaturated lipids, and basal DNAs and RNAs. Singlet oxygen will attach with basal DNAs which will change the structure of basal DNAs of cancer cells. This structure change will disturb the metabolism process of the cancer cells making them unable to grow well [11].

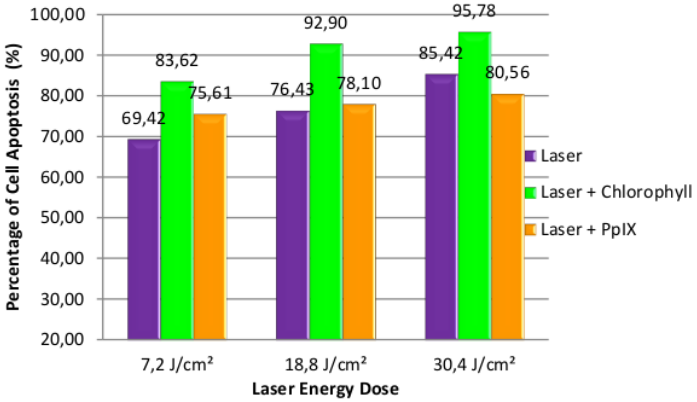


Figure 9. The Graph of the Percentage of Cell Apoptosis after Treatment.

4. Conclusions

Based on the toxicity test, the concentration of chlorophyll photosensitizers which is not toxic to cancer cells is 300 ppm with a living cell percentage of 97.6% and 350 ppm for the PpIX

photosensitizers with the percentage of living cells reaching 75.9%. The percentage cell death is the biggest on the red He-Ne laser output energy dose of 30.4 J/cm^2 which is 85.81% (without a photosensitizer addition), 96.25% (with a chlorophyll addition), and 81.62% (with the Protoporphyrin IX photosensitizer addition). The optimum energy dose of the red He-Ne laser to obtain apoptosis cell death is 30.4 J/cm^2 with cell apoptosis reaching 95.78% (chlorophyll addition) and 80.56% (with the Protoporphyrin IX photosensitizer addition).

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