



Effects of 650 nm Diode Laser and 532 nm Frequency-Doubled Q-Switched Nd:YAG Laser on The Growth of *Candida albicans*, With and Without Photosensitizers

Intisar K. Kazem and Amel M. Maki

Institute of Laser for Postgraduate Studies, University of Baghdad, Baghdad, Iraq

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Abstract: This work describes an experimental setup to evaluate the photodynamic toxicity of 650 nm diode laser and 532 nm Frequency-doubled Q-Switched Nd:YAG laser on the growth of *Candida albicans* as well as the potential fungicidal effect when combining the laser irradiation with specific photosensitizers namely methylene blue, toluidine blue, acridine orange and safranin O. In this study the findings showed that the number of colony-forming units per millilitre (CFU/ml) of *C. albicans* decreased with increasing exposure time. In particular in the case of the frequency doubled Nd:YAG laser combined with safranin O, the best lethal effect occurred at 11 minutes exposure time with 2.26 J/cm² energy density (89.18% reduction) in comparison with the group treated with neither the laser nor with the photosensitizer. Irradiation with the frequency doubled Nd:YAG laser in the presence of acridine orange had less effect in reducing the number of CFU/ml for *C. albicans*. The highest reduction (85.88%) was achieved with 2.26 J/cm² energy density at 9 min exposure time. On the other hand, the best performance in the case of diode laser was when combining with methylene blue at 28 minutes exposure and 0.58 w/cm² power density (45.38%). In the case of diode laser with toluidine blue, the highest reduction of CFU/ml (67.93%) occurred at 35 min at the same power density

Introduction

Candidiasis is one of the most frequent mycotic complications encountered for many years (Hazan, et al., 1953). *Candida albicans* is the yeast pathogen most frequently isolated from patients with vaginitis (2. Heelan, et al., 1996). There are more than 20 species of *Candida*, the most common being *Candida albicans*. These fungi live on all surfaces of human bodies. Under certain conditions, they can become so numerous that they cause infections, such as vaginal yeast infections, thrush (oral infection), skin and nailbed infections.

A promising therapeutic modality for the inactivation of pathogenic microorganisms is photodynamic Therapy (Jori, et al., 2006). Photodynamic therapy has been presented as a new antimicrobial treatment modality (Munin,

et al., 2007). In this modality light-activated drugs are used to treat diseases ranging from cancer to age-related macular degeneration and antibiotic resistant infection. The process requires the use of a chemical compound denominated photosensitizer (PS). The application of a light that corresponds to the absorption band of PS and the presence of oxygen, promotes the formation of reactive species, such as singlet oxygen (Niemz, 2004 and Stylli, et al., 2004).

The phototoxicity of laser light is possible with known photosensitizers against the yeast *C. albicans*. The literature is rich in the field of laser microbial phototoxicity as abovementioned. Nevertheless various conditions are still not completely investigated regarding the photosensitizer, laser type, and dose parameters of the laser light irradiation.

The aim of the study was to evaluate the combined effect of photosensitization of four different photosensitizers (methylene blue, toluidine blue, acridine orange and safranin O) and two different lasers (diode laser and Nd:YAG laser) on the viability of *C. albicans* and to compare between them. The work was carried out under different experimental conditions regarding the two laser parameters.

Materials and Methods

The yeast used throughout this study was *C. albicans*. High vaginal swab (HVS) samples were taken from women with candidiasis using sterile cotton tipped swap. The yeast was maintained in sterile 10% KOH solution and then subcultured on Sabouraud dextrose agar (SDA). The materials were incubated aerobically at 37°C for 48 hrs.

C. albicans was identified using microscopic, cultural and biochemical methods in addition to germ tube and chlamedospore formation tests. Biochemical tests involved sugar fermentation and Api candida tests (Milne, 1996). Specific dyes namely methylene blue, toluidine blue, acridine orange and safranin O were considered in our work as photosensitizers. Standardized suspensions (10^6 viable cells/ml) of *C. albicans* (one isolate) were prepared. Aliquot of 50 μ l of this suspension was mixed with 50 μ l of each dye solution in sterile appendrof tube for 15 min and then irradiated. Temperature was measured before and during irradiation using non-contact infrared thermometer (UT 300 Series).

C. albicans samples were treated with 0.1 mg/ml methylene blue, toluidine blue, acridine orange and safranin O solutions and then irradiated with two lasers with their specific photosensitizers at different exposure times.

The two laser systems used in the experiment were 650 nm CW diode laser and 532 nm frequency-doubled Q-switched Nd:YAG laser. After irradiation, serial dilutions 10^{-1} - 10^{-4} were prepared, and then an aliquot of 100 μ l of the irradiated suspension was spread over the surface of agar plates for each dilution. Plates were then incubated aerobically at 37°C for 48hrs until the growth was visible. Ten replicates were used for each assay. The irradiation experiments were done in dark room.

A total of 2000 assays were carried out, 400 assays for diode laser and 1600 assays for Nd:YAG laser. For each laser, the irradiation experiments involved the following four groups:

- Group I (L-P-): this group was considered as a control group. It was not subjected to laser irradiation or treated with a photosensitizer.

-Group II (L-P+): this group was treated with 0.1mg/ml of each photosensitizer with no laser irradiation.

-Group III (L+P-): this was the one that treated with laser radiation only without adding photosensitizer.

-Group IV (L+P+): this group was irradiated with laser light in the presence of a photosensitizer.

The number of colony forming units per millilitre (CFU/ml) were analysed using the SPSS statistical software package and Microsoft Office Excel, The results were log-transformed and analyzed by Analysis Of Variance (ANOVA) followed by post-hoc Tukey test. P values < 0.05 were considered significant (Negi, 2008).

Results

Fig.1 shows the results in the case of the diode laser with methylene blue. Group L+P+ (irradiated with laser in the presence of methylene blue) showed a significant reduction ($p < 0.05$) in the value of log CFU/ml compared with those of groups L-P- and L-P+ where no statistical significance differences were detected. The result mentioned was consistent throughout various exposure times of 21, 28 & 35 minutes. This indicates that the irradiation with diode laser in the presence of methylene blue was able to reduce the viability of *C. albicans*.

For toluidine blue, Fig. 2 shows the combined effect of diode laser irradiation with toluidine blue on the viability of *C. albicans*. It is clearly shown that the mean number of log CFU/ml is decreasing with increasing exposure time; significant reduction ($p < 0.05$) in log CFU/ml was achieved at 35 min. exposure time in relation to 7,14 and 21 minutes exposure time.

In the case of the combination of frequency doubled Nd:YAG laser and acridine orange, Fig. 3A shows the mean value of log CFU/ml of *C. albicans* for the four experimental groups (L-P-, L-P+, L+P- and L+P+) at different exposure times (3, 5, 7,9 and 11min) under 1.41J/cm² energy density. Considering the case where both the laser irradiation and the photosensitizer (group L+P+) are involved in the interaction, the findings indicate significant ($p < 0.05$) reduction. The maximum reduction throughout all trials

recorded the best value of log CFU/ml of 7.08 at 11 minutes.

Fig. 3B shows the mean value of log CFU/ml of *C. albicans* for the four experimental groups (L-P-, L-P+, L+P- and L+P+) at different exposure times (3, 5, 7, 9 and 11min) under 1.69 J/cm² energy density. In the case where both the laser irradiation and the photosensitizer (group L+P+) are involved in the interaction, the findings indicate significant ($p < 0.05$) reduction. The maximum reduction throughout all trials recorded the best value of log CFU/ml (6.79) at 7 minutes. We think that no saturation is being reached with the addition of the photosensitizer within our exposure times range. We may conclude with reasonable certainty that an optimum exposure time is arising and above or below that time the performance deteriorates. Statistically comparing this irradiation Fluence with previous one we found significant difference ($p < 0.05$) indicating better performance with 1.69 J/cm².

Fig. 3C shows the mean value of log CFU/ml of *C. albicans* for the four experimental groups (L-P-, L-P+, L+P- and L+P+) at different exposure times (3, 5, 7, 9 and 11min) under 1.98 J/cm² energy density. Group L+P+ showed a clear significant reduction ($p < 0.05$) with increasing exposure time. The maximum reduction in log number of CFU/ml reached 6.54 at 11 minutes.

Fig. 3D shows the mean value of log CFU/ml of *C. albicans* for the four experimental groups (L-P-, L-P+, L+P- and L+P+) at different exposure times (3, 5, 7, 9 and 11min) under 2.26 J/cm² energy density. Same behaviour as above cases was observed regarding group L-P- and group L-P+. The same is applied with laser only (group L+P-). The minimum log CFU/ml value 6.71 reached at 9 minutes exposure time. As for the case where both the laser irradiation and the photosensitizer (group L+P+) are involved in the interaction, the findings indicate significant ($p < 0.05$) reduction with values significantly better than the previous cases. The overall minimum value of log CFU/ml reached 6.48 at 9 minutes exposure time. The results of the combination of 532 nm frequency doubled laser and safranin O are shown in Fig. 4A at different exposure times 3, 5, 7, 9 and 11minutes at 1.41 J/cm² energy density. In the case of both the laser irradiation and the safranin O photosensitizer (group L+P+) the findings indicate significant ($p < 0.05$) reduction at 5, 7, 9 and 11 minutes exposure times compared with

other groups. The maximum reduction throughout all trials recorded the best value of log CFU/ml (6.45) at 11 minutes exposure times (Fig. 4A). Fig. 4B shows the mean value of log CFU/ml of *C. albicans* for the four experimental groups (L-P-, L-P+, L+P- and L+P+) at different exposure times (3, 5, 7, 9 and 11min) under 1.69 J/cm² energy density. The group L+P- showed significant difference ($p < 0.05$) at 7 and 9 minutes exposure time compared with first two groups. Combining both effects (group L+P+) the findings indicate significant ($p < 0.05$) reduction at 5, 7, 9 and 11 minutes exposure time with the maximum reduction recorded (log CFU/ml 6.58) at 11 minutes exposure time. Fig. 4C shows the mean value of log CFU/ml of *C. albicans* for the four experimental groups (L-P-, L-P+, L+P- and L+P+) at different exposure times (3, 5, 7, 9 and 11min) under 1.98 J/cm² energy density. Once more Group L+P+ showed a clear significant reduction ($p < 0.05$) with increasing exposure time. The maximum reduction in log CFU/ml reached 6.54 at 11 minutes.

Fig. 4D shows the mean value of log CFU/ml of *C. albicans* for the four experimental groups (L-P-, L-P+, L+P- and L+P+) at different exposure times (3, 5, 7, 9 and 11min) under 2.26 J/cm² energy density. Group L-P+ showed significant differences ($p < 0.05$) as well as Group L+P+ compared with other group throughout all time exposure at 2.26 J/cm² energy density. In addition to that group L+P+ showed significant difference ($p < 0.05$) in comparison with group L+P- recording the best performance in particular at 11 minutes exposure time corresponding to 6.39 in there absolute scale.

Discussion and Conclusion

In our study the highest reduction in log CFU/ml after PDT was observed in the presence of toluidine blue, followed by methylene blue. It could be noticed that the photo activation of methylene blue by the red laser radiation at 650 nm at 7 and 14 minutes presented lower fungicidal effect against *C. albicans* under study. It is worth noting that despite the different irradiation conditions in our trials the results are in reasonable agreement with those of various studies (Munin, et al., 2007, Souza, et al., 2009 and Nikawa, et al., 2003). The latter investigated the effect of red light-emitting diode on cell growth of *C. albicans*, *Staphylococcus aureus*, *Escherichia coli* and

Artemia salina; their results showed that increasing red light-emitting diode exposure time increased the cytotoxic effect of methylene blue against bacteria, yeast and microcrustacean growth. The photoinactivation using methylene blue and toluidine blue at a concentration of 0.1 mg/ml, followed by low-power GaAlAs laser irradiation at energy densities of 15.8 J/cm², 26.3 J/cm² and 39.5 J/cm², reduced the number of log CFU/ml of *C. albicans*. The results of our study are in agreement with the results of various studies (Souza, et al., 2006, Giroldo, et al., 2009 and Usacheva, et al., 2001). Giroldo and et al. in 2009 reported a significant decrease in the growth of *C. albicans* when the photosensitizer methylene blue was combined with a diode laser (684 nm and 28 J/cm²) (Usacheva, et al., 2001).

In Fig. 5B the absorption peaks in the case of toluidine blue are stronger than the ones of methylene blue. We believe that this may interpret the higher killing rate compared with that of methylene blue. As a matter of fact looking at the numbers in that case we found the same ratio as in the case of absorption relative to the rate of killing. The higher absorption leads to more transformation of optical energy into the PS material causing possible biostimulation and probable bond breaking causing higher effect via photochemical interaction processes. Usacheva and et al. in (2001) evaluated the bactericidal efficacy of the photosensitizers methylene blue and toluidine blue combined with argon (630 nm) and diode (664 nm) lasers against different bacteria. The results demonstrated that toluidine blue showed higher bactericidal activity than methylene blue for the two lasers used. Complete inactivation of bacteria was achieved with toluidine blue at concentrations 1.5-times to 7-times lower than those of methylene blue. The authors suggest that the solubility of toluidine blue should be higher in the hydrophobic region of the membrane, and, thus, toluidine blue could interact more easily with the bacterial membrane than methylene blue. As a result, the toluidine blue concentration within the microorganism cell should be significantly higher than that of methylene blue.

The frequency doubled Nd:YAG laser showed the best results in reducing the number of CFU/ml of *C. albicans* at 9 minutes exposure time at energy density of 1.98 J/cm² (74.45% reduction). Irradiation with energy density of 2.26 J/cm² gave 68.3% reduction at 9 minutes

exposure time. No significant reduction was found at low energy densities of 1.41 and 1.69 J/cm². The second important conclusion drawn from the present work is that combining the laser radiation with photosensitization led to a very noticeable effect compared with that with laser irradiation alone. The absorption spectra of the four photosensitizers (methylene blue, toluidine blue, acridine orange and safranin O) are given in Fig. 5. The behaviour of the curves showed good absorption peaks at 650 nm in both methylene blue and toluidine blue as indicated by the arrows in Figures. 5A & 5B. However, in the case of toluidine blue the absorption peak at 650 nm shows a slightly higher value by an amount around 15%.

In the case of acridine orange and safranin O. Strong absorption peaks are obvious at 532 nm as indicated by the arrows on Figure. 5C & 5D. It is noteworthy to indicate that safranin O exhibits stronger absorption peak at the frequency doubled Nd:YAG laser wavelength, i.e. at 532 nm. This fact was applicable to both lasers combined with photosensitizer. The best reduction in the number of CFU/ml of *C. albicans* was obtained by Nd:YAG laser combined with Safranin O with energy density of 2.26 J/cm² at 11 minutes exposure time (89.18 %). The best % reduction with energy density of 1.98 J/cm², 1.69 J/cm² and 1.41 J/cm² were 84.67%, 82% and 87% respectively at 11 minutes exposure time. Combination of Nd:YAG laser with acridine orange gave less % reduction in the CFU/ml of *C. albicans*. The best reduction were 85.88% at 9 minutes exposure time with energy density of 2.26 J/cm² followed by 84.67% with energy density of 1.98 J/cm² at 11 minutes exposure time.

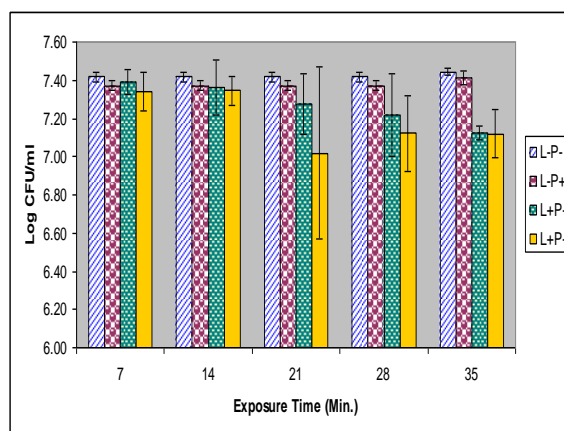


Fig. 1: Mean and standard deviation log CFU/ml obtained for Photosensitization of *C. albicans* with methylene blue using 650 nm diode laser.

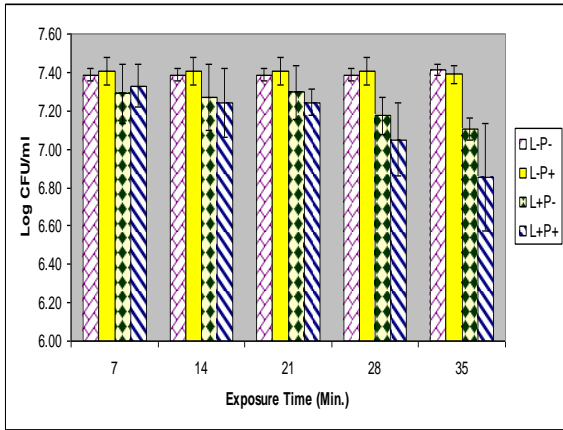


Fig. 2: Mean and standard deviation log CFU/ml obtained for photosensitization of *C. albicans* with toluidine blue using 650 nm diode laser.

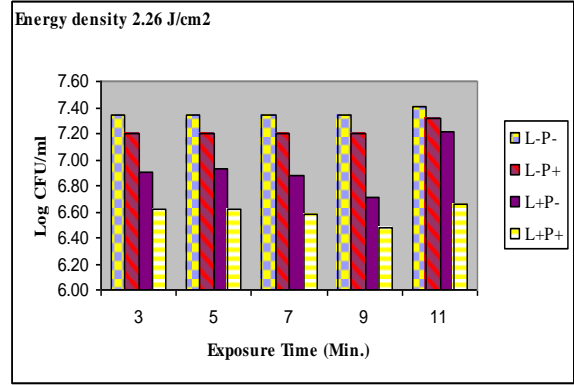
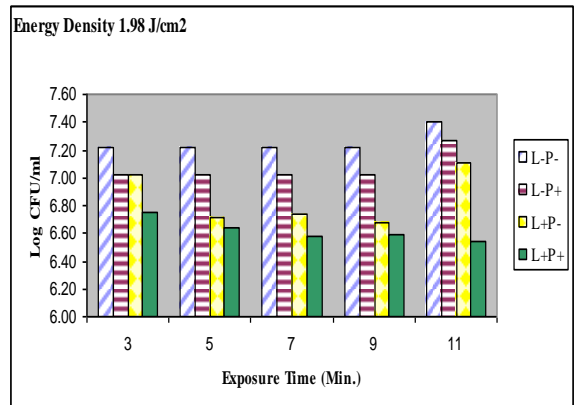
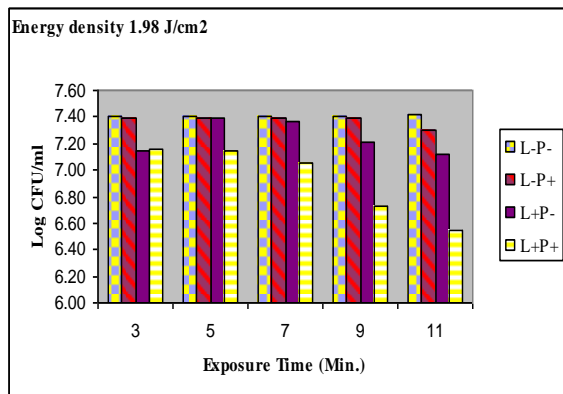
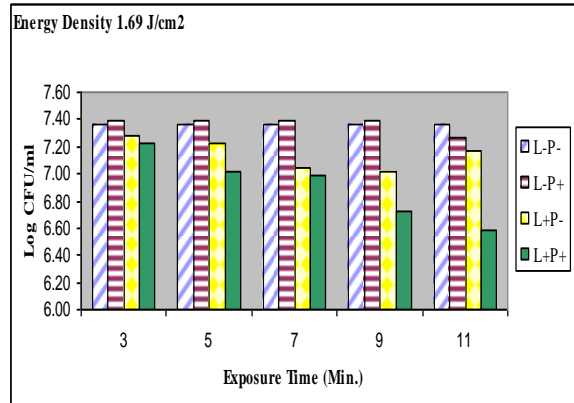
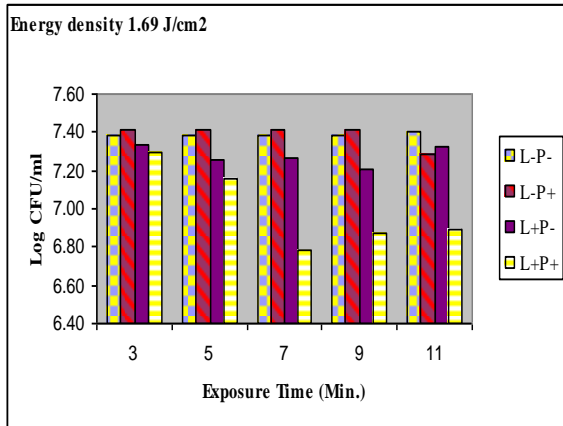
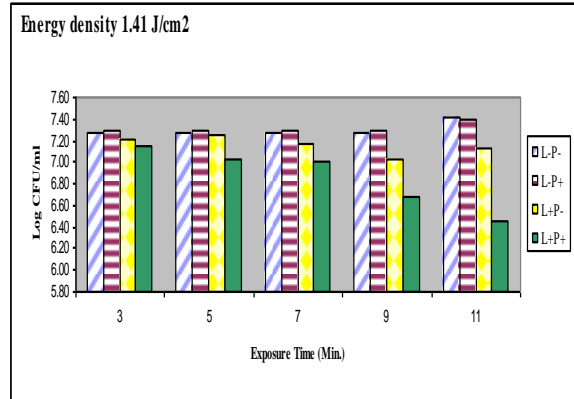
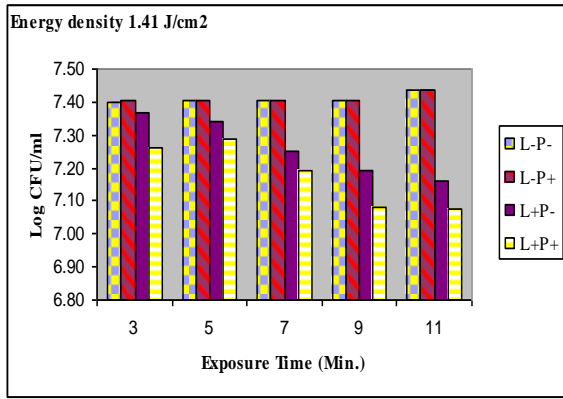


Fig. 3: Mean log CFU/ml obtained for photosensitization of *C. albicans* with acridine orange using 532 nm Frequency-doubled Q-switched Nd:YAG laser using 1.41 (A), 1.69 (B), 1.98 (C) and 2.26 (D) J/cm² energy densities.



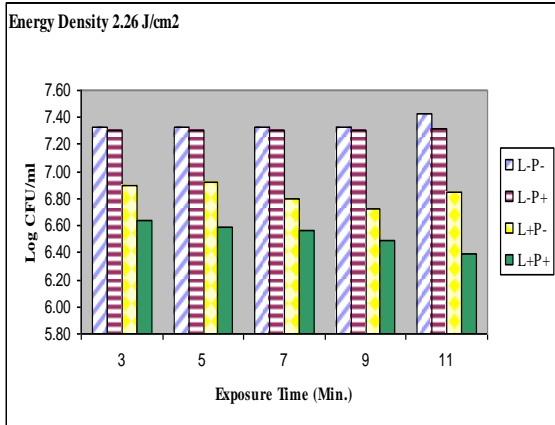
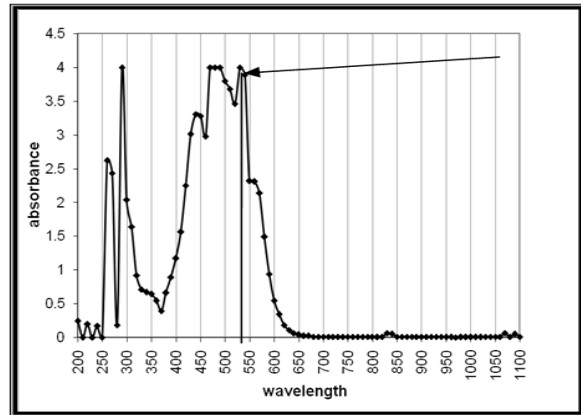
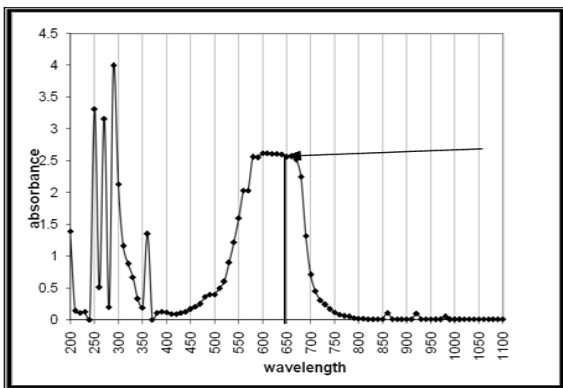


Fig. 4: Mean log CFU/ml obtained for hotosensitization of *C. albicans* with safranin O using 532 nm Frequency-doubled Q- switched Nd:YAG laser using 1.41 (A),1.69 (B),1.98 (C) and 2.26 (D) J/cm² energy densities.

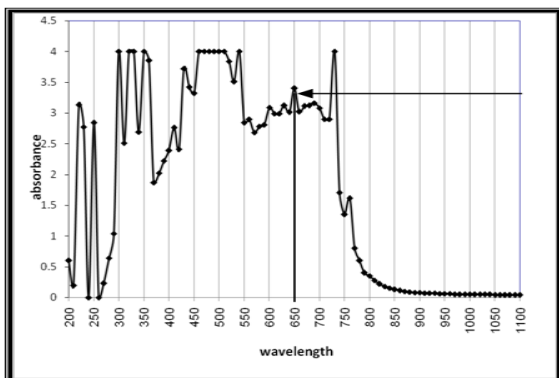


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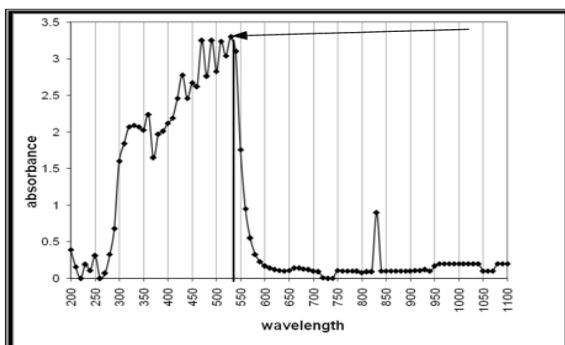
Fig. 5: Absorption spectrum of (A) methylene blue, (B) toluidine blue, (C) acridine orange and (D) safranin O.



A



B



C

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تأثير ليزر الدايدود 650 نانومتر وليزر النيديميوم ياك المفتاحي مضاعف التردد 532 نانومتر على نمو المبيضات *Candida albicans* بوجود او عدم وجود المتحسسات الضوئية

أمل مصطفى مكي

أنتصار كريم كاظم

معهد الليزر للدراسات العليا، جامعة بغداد ، بغداد ، العراق

الخلاصة : يصف هذا العمل منظومة تجريبية لتقييم السمية الفوتودينامية لليزر الدايدود (650 نانومتر) وليزر النيديميوم ياك المفتاحي مضاعف التردد (532 نانومتر) على نمو المبيضات *C. albicans* وكذلك قابلية التأثير القاتل للفطريات عند دمج اشعة الليزر مع المتحسسات الضوئية المحددة وهي صبغة المثلين الأزرق والتولودين الأزرق والأكردين البرتقالي والسفرانين O. في هذه الدراسة اظهرت النتائج بأن عدد وحدات تكوين المستعمرة في الملتتر (CFU/ml) لخميرة *C. albicans* تتضائل مع ازدياد زمن التشعيع. على الأخص في حالة ليزر النيديميوم ياك مضاعف التردد بوجود سفرانين O, ظهر افضل تأثير قاتل (89.18% نقصان) عند زمن تشعيع 11 دقيقة مع كثافة طاقة 2.26 جول/سم² بالمقارنة مع المجموعة غير المعاملة بأشعة الليزر او المتحسس الضوئي. ان التشعيع بليزر النيديميوم ياك مضاعف التردد بوجود الاكريددين البرتقالي كان له تأثير أقل في اختزال عدد وحدات تكوين المستعمرة لخميرة *C. albicans*. تحقق اعلى نقصان بطاقة كثافة 2.26 جول/سم² بزمن تعريض 9 دقائق. من جهة اخرى تحقق أفضل أداء (45.38%) في حالة ليزر الدايدود عند دمج صبغة المثلين الزرقاء عند زمن تعريض 28 دقيقة وكثافة قدرة 0.58 واط/سم². في حالة ليزر الدايدود والتولودين الأزرق تحقق اعلى نقصان (67.93%) لوحداث تكوين المستعمرة عند زمن تعريض 35 دقيقة وبنفس كثافة القدرة.