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SPIE.

Event: SPIE BiOS, 2018, San Francisco, California, United States

Photodynamic inactivation using curcuminoids and Photogem® on *Caenorhabditis elegans*

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ABSTRACT

Resistance to various anthelmintic drugs is reported in many animals and can become a severe problem for human and animal health. In this study, Photogem® and three curcuminoids compounds (curcumin, demethoxycurcumin, bisdemethoxycurcumin) were used as photosensitizers in the photodynamic inactivation (PDI) in the helminth model *Caenorhabditis elegans* to investigate the ability of this procedure to worm life cycle. Initially, the presence and location of the photosensitizers in the worm's body were verified by fluorescence confocal microscopy. Curcumin was deposited in the digestive tract and Photogem® along the body of the animal in the incubation time of 12 hours with the photosensitizer. Subsequently, a PDI procedure using a LED device was performed to illuminate the worms treated with the photosensitizers. The worms were observed by optical microscopy until 48 hours after the PDI to verify the changes in motility, the presence of eggs and larvae and the number of live worms. Curcuminoids tested separately and in combination and two light doses of 30 J/m^2 no changes were observed in the life cycle of the worm at concentrations of 2 mM and 1 mM. However, in treatment with Photogem® and a light dose of 100 J/m^2 a reduction in motility and reproduction of the worm with 0.2 mg/mL was observed after 6 hours of exposure, in addition to the death of most worms at concentrations of 6, 4, and 2 mg/mL. We suggest, therefore, that photodynamic inactivation with Photogem® may present an anthelmintic effect against *C. elegans*, but there is a need for studies on helminths with parasitic activity.

Keywords: photodynamic inactivation; anthelmintic; *Caenorhabditis elegans*; Curcuminoids; Photogem; confocal microscopy

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1. INTRODUCTION

Infections with parasitic helminths reach about 1.5 billion people worldwide and are linked to high morbidity and mortality, especially in underdeveloped areas, as well as being a burden on human health systems.[1] In addition to people, parasitic helminths also affect animals and plant crops causing substantial economic losses in the livestock and agriculture sectors. However, increasing resistance to anthelmintic drugs is a major global problem for combating parasitic diseases and research for new therapeutic compounds is scarce.[2,3] Thus, there is an urgent need for the identification of new anthelmintic approaches.

A practical and inexpensive way to study and screen new anthelmintic therapies is to employ the free-living nematode *Caenorhabditis elegans*.[4,5] This small worm (about 1 mm in length) is a well-studied model organism due to its characteristics, such as secure manipulation, transparency, self-fertilization, high reproductive capacity and a 3-day life cycle, this worm may be used in the laboratory as an alternative to parasite organisms with complex life cycles which require an animal host for propagation.[6,7]

C. elegans is also used as a model of oxidative stress study[8], and photodynamic inactivation (PDI) is a plausible therapeutic approach readily applicable in this organism for the study of photodynamic inactivation in worms. PDI results from the interaction of light at a specific wavelength with a non-toxic photosensitizer (PS) and the oxygen present in the tissues of organisms, producing reactive oxygen species (ROS), such as singlet oxygen, hydrogen peroxide, and hydroxide. ROS can destroy or damaging biological molecules by oxidation and inducing cytotoxicity, leading cells and tissues with an affinity for PS to death.[9,10] The advantage of the use of PDI over conventional medicaments is its ability to produce rapid and localized death of the target, in addition to no reports of induction of microbial resistance.[11]

A variety of photosensitizers of natural or synthetic origin can be tested on worms using PDI. Studies in *Schistosoma mansoni*, which causes schistosomiasis, reported that PDI with toluidine blue O photosensitizer (TBO) was

Optical Methods for Tumor Treatment and Detection: Mechanisms and Techniques in Photodynamic Therapy XXVII, edited by David H. Kessel, Tayyaba Hasan, Proc. of SPIE Vol. 10476, 1047612 · © 2018 SPIE · CCC code: 1605-7422/18/$18 · doi: 10.1117/12.2283242

able to generate damage in some tissues and interfere with worm motility *in vitro*, causing its death.[12] In this work, we use as synthetic curcuminoid compounds (curcumin, demetoxicurcumin and bis-demethoxycurcumin), which are yellow dyes naturally found in the *Curcuma longa* L. (Zingiberaceae family) rhizome with the common name of turmeric.[13] Curcuminoids are being extensively studied because of their range of pharmacological properties that include anti-inflammatory, antitumor, antimicrobial, anti-oxidant and many other.[13,14]

Another PS used was Photogem®, a violet porphyrin derived from the hematoporphyrin IX molecule present in blood hemoglobins. Photogem® has a range of applications in various biological systems, such as cancer treatment and microbial inactivation due to its characteristics, such as the absence of toxicity without the presence of light, ability to absorb various wavelengths and generation of good amounts of ROS.[15–17] However, although these photosensitizers present several biological activities, studies evaluating their anthelmintic activity via photodynamic activation have not yet been performed.

Thus, the purpose of this study was to analyze the effects of PDI using as photosensitizer curcuminoid compounds (curcumin, demetoxicurcumin and bis-demethoxycurcumin) and Photogem® for photodynamic inactivation of the nematode model *C. elegans* and to evaluate a location of curcumin and Photogem® in the worm, aiming, in this way, to contribute to a future application of PDI in parasitic diseases of socioeconomic importance.

2. MATERIAL AND METHODS

2.1 Nematode Culture

The strain Bristol N2 (wild-type) of *C. elegans* and *Escherichia coli (E. coli)* strain OP50 were kindly provided by Professor Marcelo Alves da Silva Moris at the Federal University of São Paulo (UNIFESP). Worms were maintained on 10 cm nematode growth medium (NGM) agar plates containing a lawn of *E. coli* OP50 as a food source [8]. Culture plates were maintained at 20 ºC.

2.2. Preparation of Photosensitizers

The synthetic curcuminoid compounds (curcumin, demethoxycurcumin, bisdemethoxycurcumin). Each curcuminoid compound was diluted in 4% tween 80 and 4 % DMSO (dimethylsulfoxide) to obtain the concentrations of 1 and 2 mM and 2 mM for the solution containing a mixture of the three curcuminoid compounds in the same ratio.

The photosensitizer Photogem® (Photogem LLC Company, Moscow, Russia), consisting of monomers dimers, and oligomers of hematoporphyrin derivatives, was diluted in distilled water to obtain the concentrations of 6, 4, 2 and 0.2 mg/mL.

2.3 Fluorescence Images

Was used a fluorescence inverted confocal microscope (Zeiss - LSM780, Zeiss, Jena, Germany) to determine a location of the curcumin and Photogem® within the *C. elegans* incubated with the compounds for 12 hours, with excitation at 458 and 405 nm respectively. The emission was captured in the range of 520-550 nm for curcumin and 600-700 nm for Photogem®.

2.4 Photodynamic Inactivation

Worms were transferred to a 24-well culture plate containing 1 mL of NGM in each well and incubated with 40 µL photosensitizers in the dark conditions for 12 hours at 20 ºC. The experiments were carried out in triplicate, and the worms samples were divided into 4 groups: Control group (treated only with the solvent), Irradiated group (treated only with LED), PS group (treated only with the photosensitizers) and PDI group (treated with the photosensitizers associated with LED).

It was used LED lighting system (Biotable®,USP, IFSC, São Carlos, SP)[18] in the wavelength of about 450 nm, and irradiated with energy of 35 mV/cm^2 and a two dose of 30 J/cm² in treatments with the curcuminoid compounds or with the solvent (4% tween 80 and 4 % DMSO) used for the solubilization of these solutions (PDI group and Irradiated group), or wavelength of about 630 nm and irradiated with energy of 70 mV/cm^2 and a dose of 100 J/cm² in treatments with the Photogem® compounds or with the water used for the solubilization of these solutions (PDI group and Irradiated group). Were observed by optical microscopy 6, 24, 30 and 48 hours after the irradiation to verify the changes in motility, the presence of eggs and larvae (data not shown) and the number of worms that remained alive.

2.5 Statistical Analysis

The tests were performed in triplicate and analyzed in Action Stat 3.1 software (Action Software, Estatcamp Team, San Paulo, Brazil) with a significance of 5% ($p < 0.05$). Experimental groups were compared using one-way analysis of variance (ANOVA) followed by Dunnett test for multiple comparisons between the four groups: Control, Irradiated, PS, and PDI.

3. RESULTS AND DISCUSSION

The location of curcumin was identified in the body of *C. elegans* incubated with the PS at 2mM for 12 hours. Figure 1 shows through fluorescence a significant presence of the molecule curcumin along the digestive tract (mouth, intestine, and rectum) accumulating in these structures of the worm. Thus, it is suggested that the nematode internalizes curcumin through ingestion along with the food present in the growth medium.

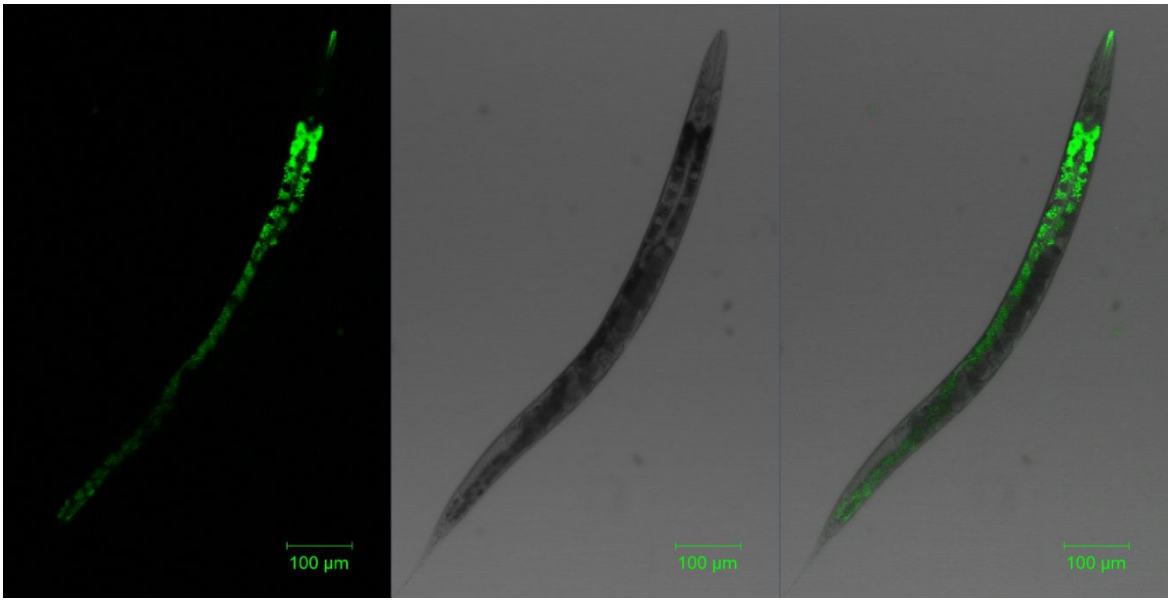

Figure 1: Fluorescence images are indicating the fluorescence of the curcumin (green color) in the digestive tract larvae of *Caenorhabditis elegans*.

Analysis of the effects of photodynamic inactivation (PDI group) on the life cycle of the worm was performed using a light-emitting LED device (Biotable®). The results of the PDI group were compared with those of the Irradiated group, PS group, and Control group.

The graphs in figure 2 show the percentage of live worms treated with curcuminoid compounds at the time of analysis. The treatments with curcuminoid compounds (PS group) at concentrations of 1 and 2 mM were not lethal to the worm as well as the control group, with several live larvae and eggs being found in the evaluated periods, and carrying an increasing number of live worms in the wells. However, treatment with light doses in both the PDI group and the Irradiated group also resulted in a growing number of live worms over time, but we observed a reduction in worm motility after 6 hours of the first dose of light dose. However, this effect was no longer observed in the 24 h period, indicating that the worms could recover from these effects. A second dose of light was applied to the worms and evaluated six hours later in the time of 30 hours after the first dose of light, and the same effect occurred again, and in the period of 48h, the motility of the worms returned to their normal state. Therefore, we suggest that this effect may be caused by the light dose of the blue LED (450 nm) and not by curcuminoid-mediated photodynamic inactivation.

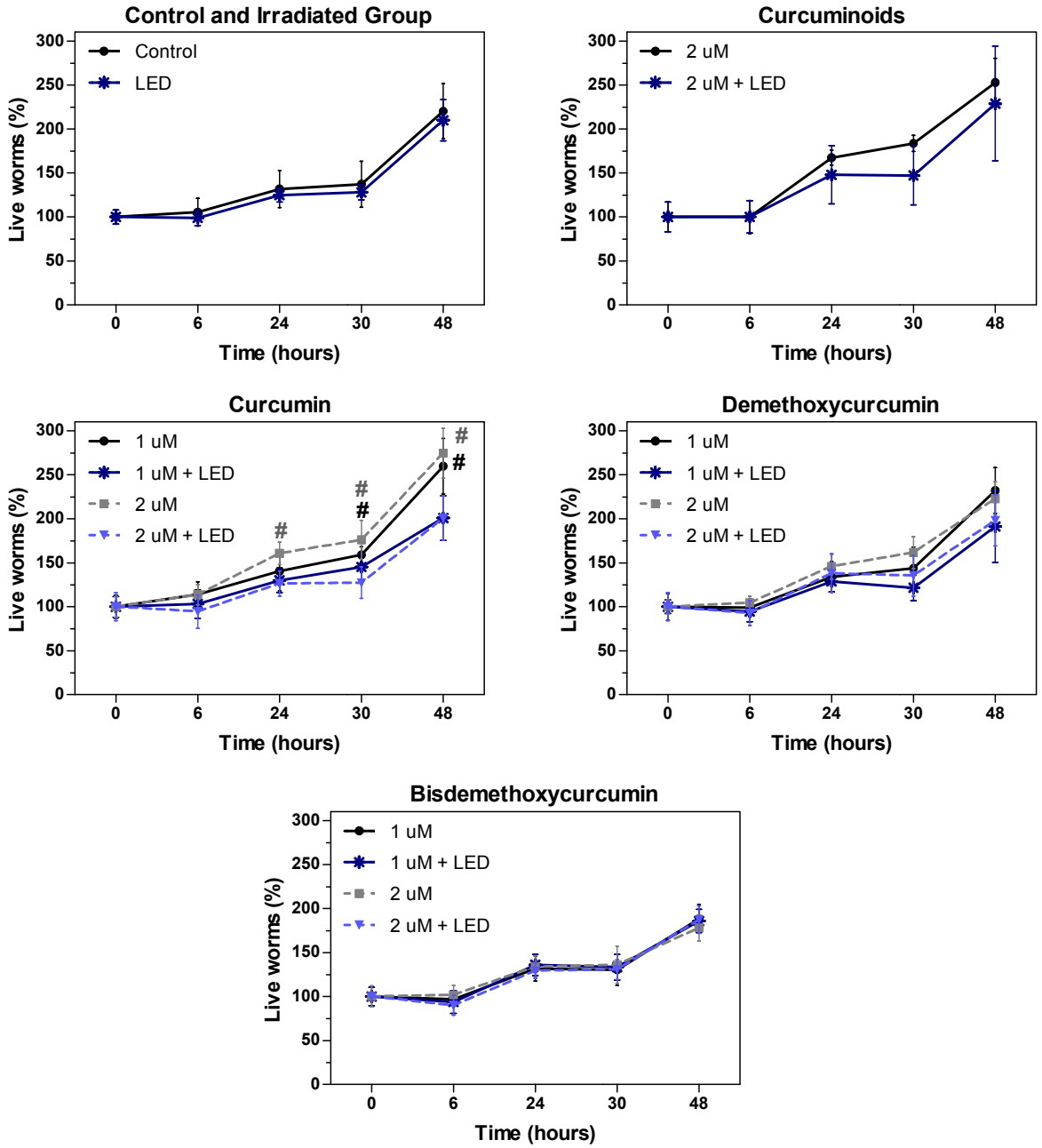

Figure 2: Percentage of live worms over the exposure periods of the Control Group, Irradiated group, PS group (curcuminoids, curcumin, demethoxycurcumin, bisdemetoxicurcumin), and PDI group (Curcuminoids, curcumin, demethoxycurcumin, bisdemetoxicurcumin associated with LED). (#) PS group (curcumin) presented statistically higher values about the Irradiated group in the marked periods of time.

The blue light has a short relative wavelength ranging from 400 to 495 nm and therefore has high energy. According to studies by Abdel-Rahman et al., the exposure for 72 hours blue light can cause oxidative stress in *C. elegans*, producing negative effects on its locomotion, survival, oviposition, besides reducing the number of eggs or delaying its maturation.[19] Thus, it is possible that the effect of the blue light (450 nm), in addition to the reduction of motility, also influenced to a smaller amount of worms for PS curcumin, demetoxicurcumin and the curcuminoid mixture of PDI group compared to its referent PS group, but not statistically confirmed. However, it is desirable that the nematicidal effect is caused by the association between the photosensitizer and light, producing a more controlled effect on only one localized target capable of retaining the PS.

In addition to curcuminoids, we also studied Photogem® as a photosensitizer in *C. elegans*. Figure 3 shows the worm incubated with 2 mg / mL PS for 12 hours. Photogem® fluorescence was found throughout the body of the worm, indicating that the photosensitizer is more distributed in the nematode, unlike curcumin that occurred more localized in the digestive tract.

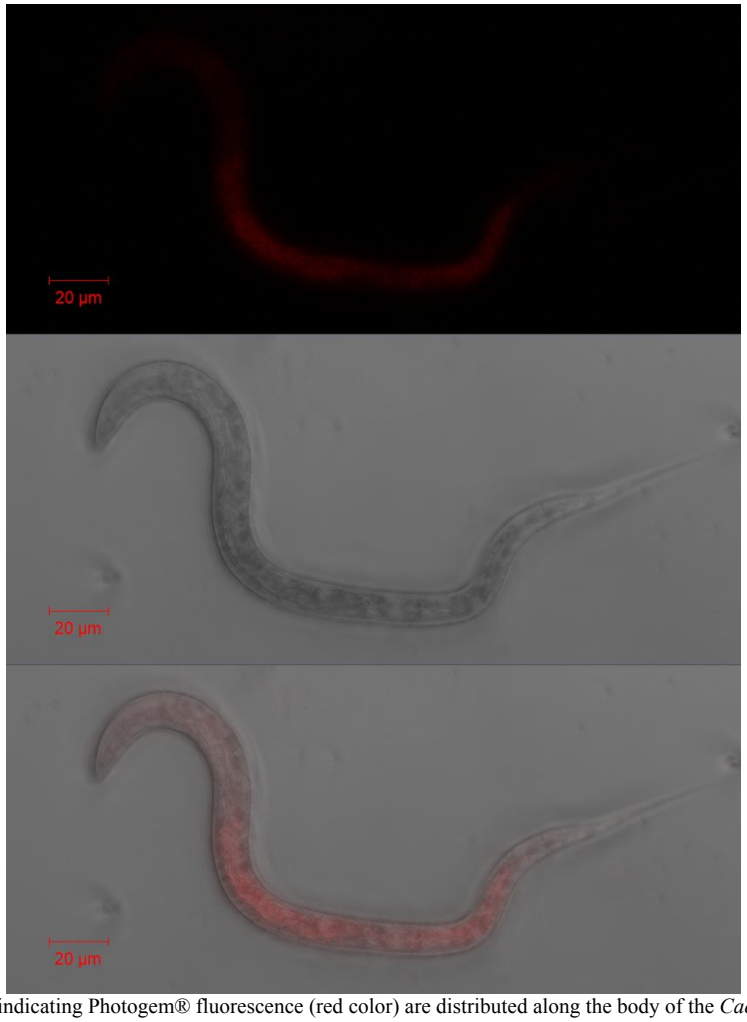

Figure 3: Fluorescence images indicating Photogem® fluorescence (red color) are distributed along the body of the *Caenorhabditis elegans*.

The graphs in figure 4 show the percentage of live worms treated with various concentrations of Photogem® at the time of analysis. The treatment of worms with Photogem® (PS group) or Irradiated group was similar to the Control group, with the presence of eggs and larvae in the analyzed periods and the increasing number of live worms over the analyzed times. However, red light (630 nm) treatment associated with Photogem® (PDI group) at concentrations of 6, 4 and 2 mg/mL was able to kill almost all worms after 6 hours of light application. At the concentration of 0.2 mg/mL of PS, the number of worms remained more constant at the time, with only a few dead worms and absence of young larvae despite the presence of eggs. Also, photodynamic inactivation mediated by porphyrin at all concentrations was able to cause a reduction in the motility of the worms that survived until the 24-hour period of application of the dose of light. This result suggests that Photogem®-mediated PDI was efficient for photodynamic inactivation of *C. elegans*, causing its death or interfering with egg development.

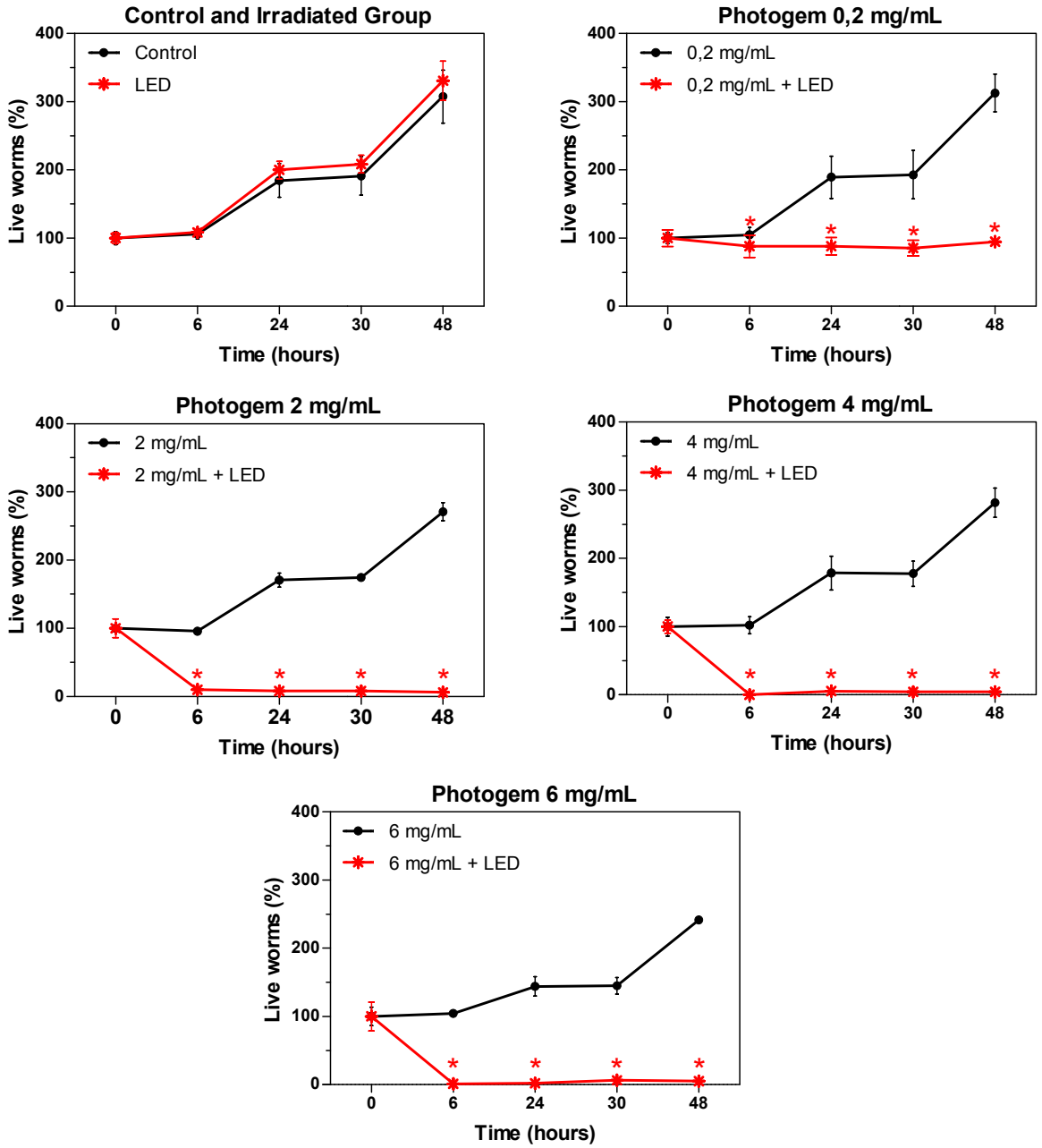

Figure 4: Percentage of live worms over the exposure periods of the Control group, Irradiated group, PS group (Photogem), and PDI group (Photogem). (*) statistical difference between PDI group and PS group, Irradiated group, and Control group.

In this way, Photogem® mediated photodynamic inactivation has an anthelmintic potential demonstrated in the *C. elegans* model. However, there is still need to verify this effect in parasitic worms. Also, some challenges still need to be overcome to carry out PDI tests for *in vivo* anthelmintic activity, such as the development of a photosensitizers delivery system and light delivery in organs affected by parasitic helminths.

4. CONCLUSION

The results showed that *C. elegans* was able to incorporate curcumin molecules into the digestive tract and to accumulate Photogem® distributed throughout its body. Still, unlike curcuminoid-mediated photostimulation, Photogem® via photodynamic activation demonstrated the potential to assist in the treatment of helminthiases, since it was able to

interfere in the lifecycle of *C. elegans*, cause their mortality and interfere with the development of eggs under the established conditions.

ACKNOWLEDGEMENTS

The authors acknowledge the support provided by Brazilian Funding Agencies: Capes; CNPq and São Paulo Research Foundation (FAPESP) grants: 2013/07276-1 (CEPOF), 2009/54035-4 (EMU), 2014/50857-8 (INCT), 2015/18745-8 (Y.R.A. scholarship).

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