Visible light enhances the antimicrobial effect of some essential oils

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Abstract

The photodisinfection is a topical, broad spectrum antimicrobial technology, targeting bacteria, virus, fungi, and protozoa effective for single cells as for biofilms. Natural molecules have been studied less than synthetic agents in the process but they are currently receiving great interest. Therefore, the aim of this study is to evaluate for the first time if non-coherent blue and red light enhances the antimicrobial activity of some essential oils when standard strains for antibiotic or fungicide tests are enlightened *in vitro*. *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Candida albicans* collection strains were irradiated with monochromatic visible light from light emitting diodes in the presence of 5% and 0.5% eucalyptus (*Eucalyptus globulus*), clove (*Eugenia caryophyllata*), and thyme (*Thymus vulgaris*) essential oils. Microbial levels were measured by plate count on culture media. In this preliminary report, the results differ according to the kind and concentration of antimicrobial oils, the wavelength of light, and the prokaryotic or eukaryotic microorganism. The results support the idea that mainly blue light enhances the innate antimicrobial activity of the essential oils, especially phenols, and could offer a very efficient and natural way to combat microorganisms in several industries and medical applications (cutaneous and oral infections, medical textiles, foodstuffs and fruit surface, etc.).

Keywords: Photodisinfection; Visible light; Essential oils; *Staphylococcus epidermidis*; *Pseudomonas aeruginosa*; *Candida albicans*

1 Introduction

Photodisinfection (PD) is a topical, broad spectrum antimicrobial technology, targeting a wide range of Gram-positive and Gram-negative bacteria, virus, fungi, protozoa, and drug-resistant microorganisms [1–6]. The PD efficacy has been demonstrated both *in vivo* and *in vitro* on simple structures as single cells but also in biofilms [7,8]. Photoreactive compounds are used together with visible light in order to active phototoxic response. Visible light disinfection is specific to only those microorganisms that absorb the substance and are exposed to a wavelength of light with sufficient radiant intensities and duration to initiate their inactivation. The red and the blue-violet range of visible light, generated through light emitting diodes (LED's), is commonly utilized to initiate a photoreaction with endogenous or exogenous molecules [1,5]. The generation of reactive oxygen species (ROS) causes general oxidative breakdown and also kills cells. The detailed mechanism of action by ROS has been widely discussed [8,9].

The nature of the compounds used in PD is very heterogeneous. There are two main groups according to their nature, synthetics and natural agents. Synthetics are the broader group and are comprised of acridines, cyanines, phthalocyanines, porphyrins, or phenothiazines [1,2,5,7,8,10]. Although different molecules from natural source have been widely studied, the current trend is the screening of raw natural products in order to detect PD activity. One recent example has been the detection of PD activity in honey [11,12]. In this scenario, it makes sense to consider other products from vegetal source as the essential oils. Nowadays, the use of essential oils extracted from plants and spices without light as antimicrobial agents is an important research area for their applications in medicine and in the cosmetic, pharmaceutical, agricultural or food industries to replace synthetic compounds [13–15]. Essential oils are the odorous, volatile products of a plants secondary metabolism, normally formed in special cells or groups of cells. They may be present in glandular cells or ducts in any or all organs of the plant including roots, stem, buds, leaves, flowers and fruits. In the literature, there is some discordance between the levels of antimicrobial activity reported for various essential oils. Data such as the geographical origin of the plants, the time of harvest, the varied chemical composition, the method of extraction, and the methodologies used to evaluate their

biological activities, among others, are basic questions to learn the antimicrobial action and compare results [16,17]. The properties of the essential oils are determined by the basic structure of the main component and its functional groups. The same plant species can have several chemotypes based on the most abundant secondary metabolite. While the total amount of essential oils among the chemotypes can show no differences, the concentration of the main component can differ significantly. The chemical structure of the main component will determine their antimicrobial activity. Mainly, essential oils are hydrocarbon terpenes (isoprenes) and terpenoids (isoprenoids). Oxygenated derivatives of hydrocarbon terpenes such as alcohols, aldehydes, ketones, acids, phenols, ethers and esters have variable antimicrobial activity.

Clove, thyme, oregano, cinnamon, and peppermint oils, among others, have been described by several authors as potent antimicrobial phenols [13,15,17–19]. Their chemical structure and charge makes their action in the PD process possible. According to our knowledge, there are no studies that use light and the essential oils to improve their antimicrobial action. Therefore, the aim of this work is to evaluate *in vitro* the antimicrobial efficacy of noncoherent visible blue and red light when essential oils are used against strains of standard microorganisms for antibiotic or fungicide testing. Hypothesis to corroborate this are: (1) bacteria are more sensitive to radiation than yeast, and (2) light with a shorter wavelength (blue) increases the antimicrobial effect of the essential oils. The use of light can improve the power of the current uses of essential oils as antimicrobials, for example during their use in cutaneous and oral infections, medical textiles, foodstuffs and fruit surface among others. Nevertheless, additional studies will be needed to extend these preliminary results.

2 Materials and methods

2.1 Essential oils

The essential oils of eucalyptus (*Eucalyptus globulus*), clove (*Eugenia caryophyllata*) and thyme (*Thymus vulgaris*) from Aromium® (Barcelona, Spain) were used. Eucalyptol, eugenol and thymol are the main active principles of eucalyptus, clove and thymus essential oils in their respective chemotypes. Chemically they are a monoterpene cyclic ether, a phenylpropane derivate, and a monoterpene phenol, respectively (Fig. 1). All of them were diluted with dimetilsufoxide (DMSO) to a concentration of 20% stock solutions and were tested at 5% and 0.5% concentration. Methylene blue at a final concentration of 50 µM was also used in the study as a positive control of light.

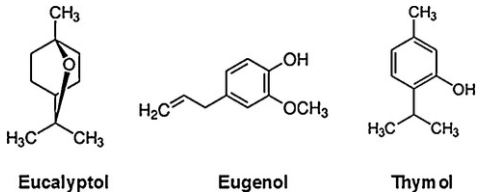

Fig. 1 Chemical structures of the main active principles of the essential oils used (eucalyptus, clove and thyme) and methylene blue.

alt-text: Fig. 1

2.2 Microbial strains and growth media

Staphylococcus epidermidis (CECT 231), *Pseudomonas aeruginosa* (CECT 110), and *Candida albicans* (1392) were employed in the study. All of them were used as standard strains for antibiotic or fungicide testing. Bacteria were cultured at 37 °C after being streaked on Tryptic Soy Agar (TSA) and yeast on Sabouraud Dextrose Agar (Scharlab, S.L., Barcelona, Spain). Overnight cultures were diluted in phosphate buffered saline (PBS) at pH 7.4 to a final concentration of 1×10^8 CFU/mL at 600 nm. These cultures were used to determine the antimicrobial effect of the essential oils and as a negative control of light.

2.3 Irradiation conditions

One ELISA plate was used to irradiate each population of microorganisms. Two different concentrations of oils were used in the study. The samples of 5% concentration were prepared by adding 250 µL of 20% essential oil to 750 µL to each inoculum. For a final concentration of 0.5% essential oils, 25 µL of each one was added to 975 µL of each inoculum. Controls with methylene blue and PBS + DMSO were prepared by adding 25 µL of 2000 µM methylene blue and 25 µL of PBS + DMSO respectively to 975 µL of each inoculum. The amount of DMSO added in the control was proportional as that used to dilute the essential oils. The same procedure was followed with unirradiated cultures to control the light antimicrobial effect (unlighted agent). Non irradiated samples were incubated for 30 min in darkness at room temperature. The corresponding samples, including the PBS + DMSO control, were irradiated with a part of visible light at 469–470 nm (blue light) and 620–630 nm (red light) for 15 min (after a dark incubation of 15 min) using a Photo Activation Universal Light (PAUL) instrument (GenIUL®, Terrassa, Spain) [11,12]. Light dose was 21.6 J cm^{-2} and 14.4 J cm^{-2}, respectively. After the treatment, the samples were centrifuged at 14,500 rpm for 5 min and the pellet was recovered and resuspended in PBS to remove any remaining antimicrobial substances. The populations of bacteria and yeast were measured by plate count on TSA or Sabouraud Dextrose Agar, respectively (pour plate method), after performing various dilutions, plating 100 µl in duplicate and incubating at 37 °C for 18–24 h.

Each experiment was performed in duplicate on different days.

3 Results

Results of the mean (+-standard deviation) of antimicrobial activity of the essential oils on *S. epidermidis* (CECT 231), *P. aeruginosa* (CECT 110) and *C. albicans* (CECT 1392) after irradiation with blue and red light, and unirradiated cultures are summarized in Table 1 and Fig. 2.

Table 1 Antimicrobial activity of the essential oils and methylene blue on *S. epidermidis* (CECT 231), *P. aeruginosa* (CECT 110) and *C. albicans* (CECT 1392) populations after irradiation with blue and red light and unirradiated cultures. Mean of microbial population is expressed in log CFU/mL. Limit of detection (LD) is 10 CFU/mL. The reduction of the populations higher than 3 logs compared to unenlightened cultures are in bold.

alt-text: Table 1

Microorganism	Essential oil	Blue light	Red light	No light	Lighted PBS + DMSO
S. epidermidis	5% Eucalyptus	1.40E + 05	6.70E + 06	4.03E + 07	4.90E + 06
	0.5% Eucalyptus	2.70E + 08	2.87E + 08	2.90E + 08	2.98E + 08
	5% Clove				3.17E + 08
	0.5% Clove			3.50E + 03	1.14E + 07
	5% Thyme				
	0.5% Thyme		8.10E + 06	3.40E + 06	2.80E + 07
	Methylene blue	6.30E + 07	6.00E + 05	8.35E + 07	3.17E + 07
P. aeruginosa	5% Eucalyptus	9.62E + 04	2.49E + 07	7.76E + 07	2.23E + 08
	0.5% Eucalyptus	3.42E + 08	3.40E + 08	3.48E + 08	3.51E + 08
	5% Clove			2.46E + 04	2.47E + 08
	0.5% Clove			1.67E + 05	3.47E + 06
	5% Thyme				7.43E + 07
	0.5% Thyme				1.17E + 08
	Methylene blue	3.38E + 07	1.10E + 06	4.01E + 07	2.47E + 07
C. albicans	5% Eucalyptus	7.26E + 04	5.30E + 05	5.51E + 05	6.46E + 05
	0.5% Eucalyptus	2.82E + 07	2.90E + 07	3.10E + 07	3.24E + 07
	5% Clove				6.18E + 07
	0.5% Clove	2.33E + 04		1.16E + 05	1.26E + 07
	5% Thyme				4.51E + 07
	0.5% Thyme	3.95E + 06	5.25E + 06	5.88E + 06	2.99E + 07
	Methylene blue	3.00E + 07	2.20E + 05	6.20E + 07	6.18E + 07

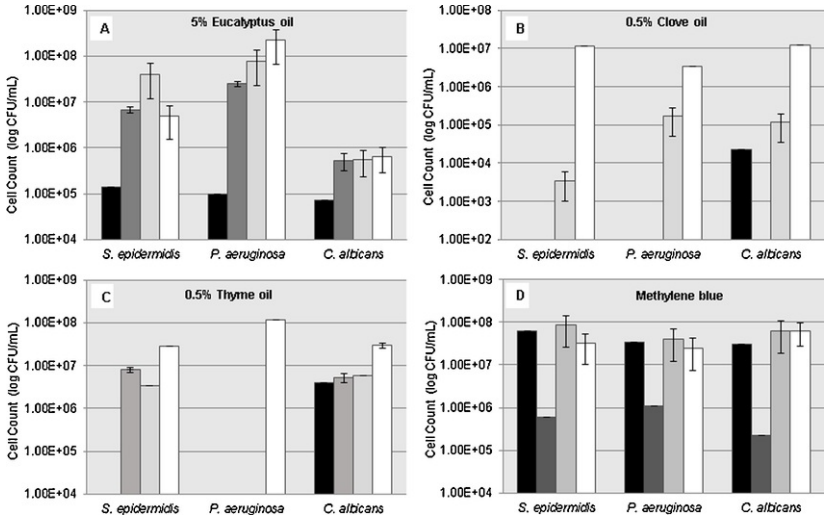

Fig. 2 Mean (±standard deviation) of *S. epidermidis* (CECT 231), *P. aeruginosa* (CECT 110) and *C. albicans* (CECT 1392) populations after treatment with 5% eucalyptus oil (A), 0.5% clove oil (B), 0.5% thyme oil (C) and methylene blue (D). ■ Blue light with agent, ■ red light with agent, ■ unlighted agent, □ lighted PBS + DMSO.

alt-text: Fig. 2

The results showed the extent of killing was dependent on the kind and concentration of essential oil, the light wavelength of light, and the microorganism. Light produced no antimicrobial effect when samples were illuminated without essential oil. In addition, the effect of both lights on the viability of cells in the presence of DMSO was also evaluated. The results did not show reduction in cell viability in any of the microorganisms tested (data not shown). Therefore, only results of blue light are shown in the table and in the figure.

Eucalyptus—The results of the eucalyptus oil (Fig. 2A) showed different antimicrobial activity according to the concentration of the essential oil and the wavelength of light, but in general was low. 5% eucalyptus oil without light treatment slightly reduced the population of the gram-negative bacteria when it was compared with the PBS + DMSO control. No effect of the oil was observed on the yeast and an increase of the gram-positive population was present. The treatment with blue light was more effective against all microorganisms than red light. The comparison of results between lighted and unlighted microbial populations showed that blue light was especially effective against *P. aeruginosa* (almost 3 log reduction), followed by *S. epidermidis* (more than 2 log reduction) and finally *C. albicans* (almost 1 log reduction). Red light practically had no effect on populations. The 0.5% concentration of the eucalyptus oil had no remarkable effect on any microorganism with or without light.

Clove—The extent of killing with the clove essential oil (Table 1) was dependent both on the light energy delivered and the photosensitizer concentration but in a different sense than the eucalyptus oil. Clove is a more potent antimicrobial agent. Therefore, the concentration of 5% completely eliminated the population of the *S. epidermidis* and *C. albicans*, and a 4 log reduction in respect to PBS + DMSO concentration on *P. aeruginosa* was observed. Hence, it was not possible to check the antimicrobial effect of light and a concentration of the oil ten times lower was used. 0.5% clove oil (Fig. 2B) led an important diminution of the viability of the three microorganisms when the results were compared with the PBS + DMSO control. A reduction of the *S. epidermidis* population of about 4 logs was achieved while for *P. aeruginosa* and *C. albicans* it was greater than 1 log. Light produced a very high decline of the microbial populations. In this case, both wavelengths showed high antibacterial power. A slight reduction of the yeast compared with the unlighted population was observed when blue light was used, while the complete elimination of the yeast with red light was observed.

Thyme—The results of 5% thyme essential oil also led to the elimination of the microorganism (Table 1). Therefore, a lower concentration was needed to discover the antimicrobial effect of light on cell viability. The population of *P. aeruginosa* was also eliminated when 0.5% thyme oil was used (Fig. 2C) and therefore, an 8 log reduction without light was shown. A minor effect was detected on *S. epidermidis* and on *C. albicans* in which the reduction of the population was lower than 1 log when the results were compared to PBS + DMSO control. Blue light killed the entire population of the gram-positive bacteria and a decrease of almost 7 logs was observed in comparison with unlighted thyme oil. This wavelength of light had no effect on the yeast. Red light did not potentiate the antimicrobial effect of the oil or the bacteria or the yeast.

Methylene blue − The results of methylene blue are shown in Fig. 2D. The chemical substance was effective against the microbial populations only with red light. In this sense, a reduction of between 1 and 2

logs of the viable cells of the microorganisms in respect to PBS + DMSO concentration was observed.

4 Discussion

The present study set out to probe the antimicrobial efficacy of some essential oils when populations were illuminated with blue and red light. The activity of essential oils is related to the respective composition, the structural configuration of the constituent components of the volatile oils and their functional groups, and the possible synergistic interactions between components [17]. In the literature, some studies reported that components with phenolic structures, such as eugenol and thymol are known to possess some antimicrobial activities as bactericidal or bacteriostatic agents against gram-positive and gram-negative bacteria, and also against yeasts [13,14,17–19]. In this job, the analysis of the antimicrobial activity on the microorganisms of the unlighted agents confirmed that phenols (eugenol and thymol) were the most potent antimicrobial agents. 5% clove and thyme essential oils showed greater antimicrobial activity that 5% eucalyptus oil and led to the complete elimination of microbial populations. Thus, 0.5% concentration of clove and thyme oils produced a decrease in the viability of the cells much greater than 5% eucalyptus oil especially on bacteria. In the current study, thyme oil was especially effective against *P. aeruginosa* when the population was not illuminated producing their total elimination. This result disagrees with Mayaud et al. [19], which asserts that *P. aeruginosa* was the bacteria which was most highly resistant to the essential oils containing phenol. The presence of an external membrane is particularly impermeable to essential oil molecules and the presence of efflux mechanisms and porine-dependent inhibition, protecting the bacteria against the action of oils. Observations with a confocal laser microscope confirmed that thymol disrupts the cell membrane in *P. aeruginosa* and *S. aureus* because it acts at the phospholipid bilayer of the cells [20]. The action of the phenols on yeast was not remarkable in the present study, contrary to previous works [21] that confirm the higher susceptibility of *C. albicans* to the thyme and clove oils because they may break up its structural integrity faster than the bacteria. The different results of the antimicrobial sensitivity could be due to several factors. The strains of microorganisms, the concentration and composition of the essential oils and the test methods used appear to be important factors on reproducible results. Methylene blue caused no effect on microbial populations, as expected.

The novelty and importance of the present study is related to the action of light with essential oils. The results reveal that the efficacy of light to produce the death of the cells depends on the molecular structure and concentration of the essential oil, the wavelength of light and the type of microorganism (prokaryotic or eukaryotic).

Regarding the essential oils and microorganisms, the clove and thyme oils were the more potent antimicrobial agents when illuminated. The good results, especially on bacterial viability, obtained with these unlighted phenols were increased with light. Small concentrations of the oils led to large cell death when light struck them. As with the first hypothesis, the microorganisms, particularly bacteria, were sensitive to light. Blue light was more effective than red, thus confirming the second hypothesis. The essential oils together with blue light always led to the decrease of viable cells in different magnitudes depending on the specie. In many cases complete removal of bacterial populations was obtained. These results are agree with those of Birmpa et al. [6] which confirm that visible light particularly at wavelengths of 405 nm is effective in inactivating gram-positive and gram-negative bacteria species and antibiotic-resistant microorganisms. The effect in the study could be due to changes in the membrane permeability of the irradiated cells. Synergistically, essential oils with antimicrobial effects has the ability to disrupt lipid structure of the cell wall of bacteria, leading to destruction of cell membrane, cytoplasmic leakage, lysis of the cell, and ultimately cell death [22]. The results of the present work indicated that if the chemical composition and concentration of the essential oils were suitable the elimination or reduction of the bacterial population would occur regardless of the character gram. Therefore, it is not possible to confirm that gram-positive bacterium was more sensitive than gram-negative bacterium to light, contrary to what previous studies with other molecules indicate [5]. The chemical structure of the phenols would allow their good absorption by bacteria and the wavelength of light would provide sufficient radiant intensities and duration to initiate the inactivation of the microorganisms. Due to the intrinsic complex nature of plant derived products, it is possible that some components of essential oils could act as photosensitizers generating ROS. Therefore, an additional oxidative cellular damage that increase the well known antimicrobial effect of these products could occur.

C. albicans were more difficult to kill than bacterial cells. The different kinds and concentrations of the essential oils produced minimal decrease of the yeast populations except when 0.5% clove oil and red light was used. This could be explained by the poor absorption of the substances and the more complex targets present in a thick external wall of the yeast [5]. According to the literature, a multi-hit process is necessary in eukaryotes, whereby saturation of more than one molecular target is required before cell death occurs [23,24].

It is important to keep in mind that many essential oils have antioxidant properties and can protect the cells against oxidative damage. The essential oils that showing different levels of cytotoxicity also exhibited different antioxidative capacities depending on the composition of the oil especially on their phenolic content [25]. Although the constituents of essential oils can act as antioxidants, they may also act as prooxidants and affect inner cell membranes. Depending on the type and concentration, this effect may result in cellular cytotoxicity [22,26]. Components of natural products especially volatile terpenes and phenolic components which show antioxidant activity, can be oxidized by ROS and thus generate additional radical species like panoxyl, hydroxyl and superoxide radicals and hydrogen peroxide and affect the cellular redox status in the so-called “antioxidative stress” [27]. Indeed, antioxidants by interacting with ROS are converted into prooxidants which are able to oxidize lipids, proteins and DNA. The in balance between the generation and depletion of ROS, a reduced activity of the protective antioxidant defense system, and a perturbation of cellular redox status generate the death of the microorganisms [28].

Methylene blue is an efficient producer of singlet oxygen required against broad-spectrum bacteria and cultures of *Candida* species although the yeast was considerably less susceptible than bacteria [5,29]. In the current work, a weak decrease in the viability of microbial cells and methylene blue was observed if the results were compared with that some essential oils. It is only remarkable the decrease in the viability of the microorganisms with red light.

The results of the study support the idea that mainly monochromatic blue light enhances the innate antimicrobial activity of the essential oils, especially phenols, and could offer a very efficient and natural way to combat microorganisms in several industries and medical applications. Decontamination could be accelerated if monochromatic visible light was used. Nowadays, some essential oils can be used in the preservation of foodstuffs against bacteria and to increase the shelf life of foodstuffs. Therefore, their illumination with visible light could facilitate this. From a medical point of view, impregnation of medical textile as bandages with essential oils and lighting could improve the reduction or complete elimination of microorganisms and the healing of wounds. The applications of the study can be numerous and varied, but the goal is always to find a natural, safe and fast method to facilitate the death of microbial cells and prevent the development of resistance to drugs.

5 Conclusions

This is the first published study that uses essential oils as photosensitizers. Results of preliminary experimental investigations demonstrate for the first time that the efficacy of PD to produce the death of the microorganisms depends on the molecular structure and concentration of the essential oil, the wavelength of light, and the type of microorganism (prokaryotic or eukaryotic). Essential oils, especially phenols, are good compounds to enhance their innate antimicrobial activity. These essential oils together with blue light always lead to a decrease of viable cells in different magnitudes depending on the specie, but both bacteria are especially sensitive to light with phenols. If the chemical composition and concentration of the essential oils are suitable, the elimination or reduction of bacterial population with blue light will occur regardless of character gram.

This study opens a new and promising research field to facilitate the removal of microorganisms with essential oils and visible monochromatic light. Therefore, additional studies with different essential oils, time of light exposure and microorganisms will be needed to confirm this preliminary data. Important future goals could include identify the active antimicrobial components of the essential oils and know how light affects to these with the use of accurate analytical chemistry tools. Likewise, the molecular mechanisms by which these components so effectively act as antimicrobials will be welcome. In this study, a small number of essential oils were sampled, but a wide range of other oils is available in nature, bearing enormous potential for the discovery of alternatives to antibiotics in conjunction with the light.

Funding

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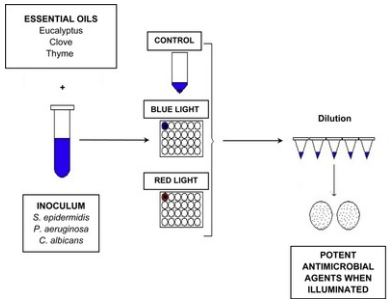
Graphical abstract



Highlights

- For the first time, antimicrobial essential oils and visible light is analysed.
- Blue light always enhances the innate antimicrobial activity of oils especially of phenols.
- Regardless of gram character both bacteria are easier to kill than yeast.
- Visible light with essential oils can be effective in several industries and medical applications.

Queries and Answers

Query: “Your article is registered as a regular item and is being processed for inclusion in a regular issue of the journal. If this is NOT correct and your article belongs to a Special Issue/Collection please contact e.rajesh@elsevier.com immediately prior to returning your corrections.”
Answer: It is correct. The article has to be processed for inclusion in a regular issue of the journal

Query: The author names have been tagged as given names and surnames (surnames are highlighted in teal color). Please confirm if they have been identified correctly.
Answer: Yes, they have been identified correctly

Query: Please check the presentation of affiliations "a, b" and correct if necessary.
Answer: The affiliations are correct

Query: Please check the hierarchy of section headings.
Answer: All is correct

Query: Please check the section "Funding" and correct if necessary.
Answer: The funding is correct

Query: Please check the presentation of "Table 1" and correct if necessary.
Answer: I think the last column of the table could be improved. The width of the column should be the same as the rest of columns. In proof I can observe a mistake in the last column heading (Ligthed PBS + DMSO) because lack the last O (... DMS). On the other hand, I have detected an error in the figure caption of Fig 2. The marks of the different columns of the histogram have different sizes.