

# Comparative singlet oxygen photosensitizer efficiency of berberine, rose bengal, and methylene blue by time course nuclear magnetic resonance (NMR) monitoring of a photochemical 4+2 cycloaddition endoperoxide formation

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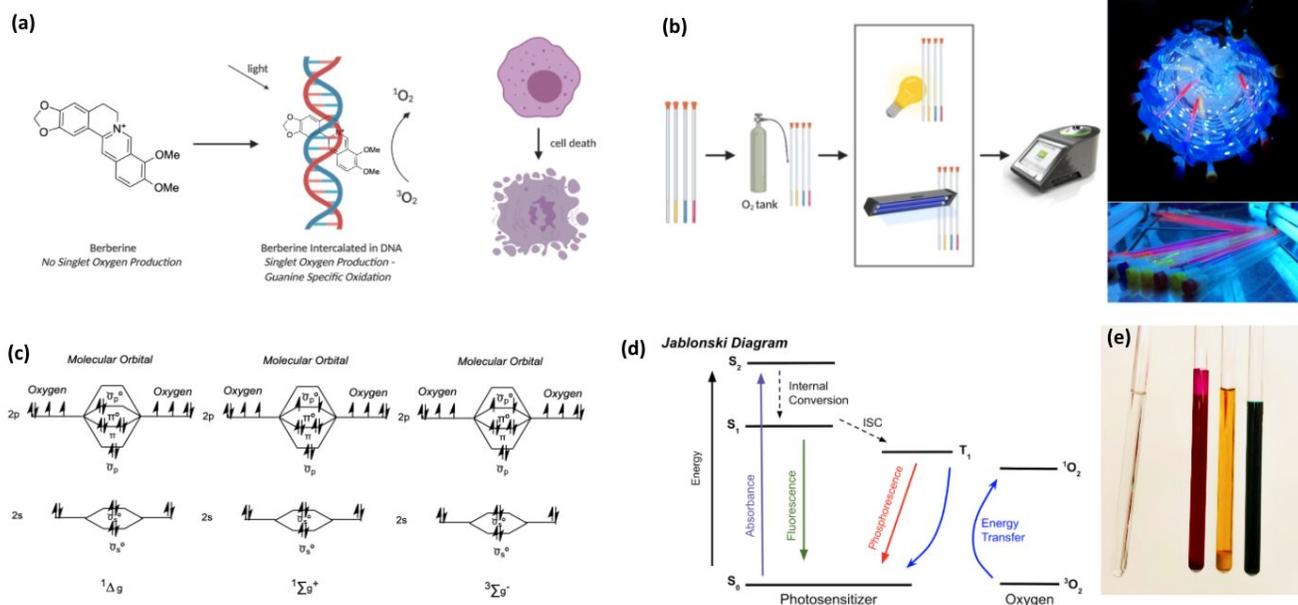
**KEYWORDS** *Berberine, Photosensitizer, Rose Bengal, Methylene Blue, Singlet Oxygen Photosensitizer*

**ABSTRACT:** Berberine, a natural product alkaloid, has been shown to exert biological activity via in situ production of singlet oxygen, a highly reactive oxygen species, when photo irradiated. Berberine utilizes singlet oxygen in its putative mechanism of action, wherein it forms an activated complex with DNA and photosensitizes triplet oxygen to singlet oxygen to specifically oxidize guanine residues, halting cell replication, leading to cell death. This has potential application in photodynamic therapy, alongside other such compounds which also act as photosensitizers and produce singlet oxygen in situ. The quantification of singlet oxygen in various photosensitizers, including berberine, is essential for determining their photosensitizer efficiencies. We hypothesized that the singlet oxygen produced by photoirradiation of berberine would be superior to the aforementioned photosensitizers when irradiated with UV light, but inferior under visible light conditions, due to its strong absorbance of UV wavelengths. Here, we report the usage of time course <sup>1</sup>H nuclear magnetic resonance (NMR) spectroscopy to trap singlet oxygen via a 4+2 cycloaddition with terpinene, as well as theoretical calculations by time-dependent density functional theory (TD-DFT) towards quantification berberine's singlet oxygen production against two known photosensitizers, methylene blue and rose bengal, to determine berberine's efficacy as a singlet oxygen photosensitizer. We envision that similar processes can be utilized for the evaluation of berberine analogs or other photosensitizing agents, and the identification of other potential medicinally significant singlet oxygen photosensitizers.

## Introduction

Berberine is a naturally occurring isoquinoline alkaloid derived from plants of the genus *Berberis*, whose use has been documented in ancient medicine as early as 3000 B.C. to treat various diseases such as diabetes, infections, and inflammation (1). Berberine has been indicated to possess various medicinal characteristics ranging from antimicrobial to anticancer properties (2, 3).

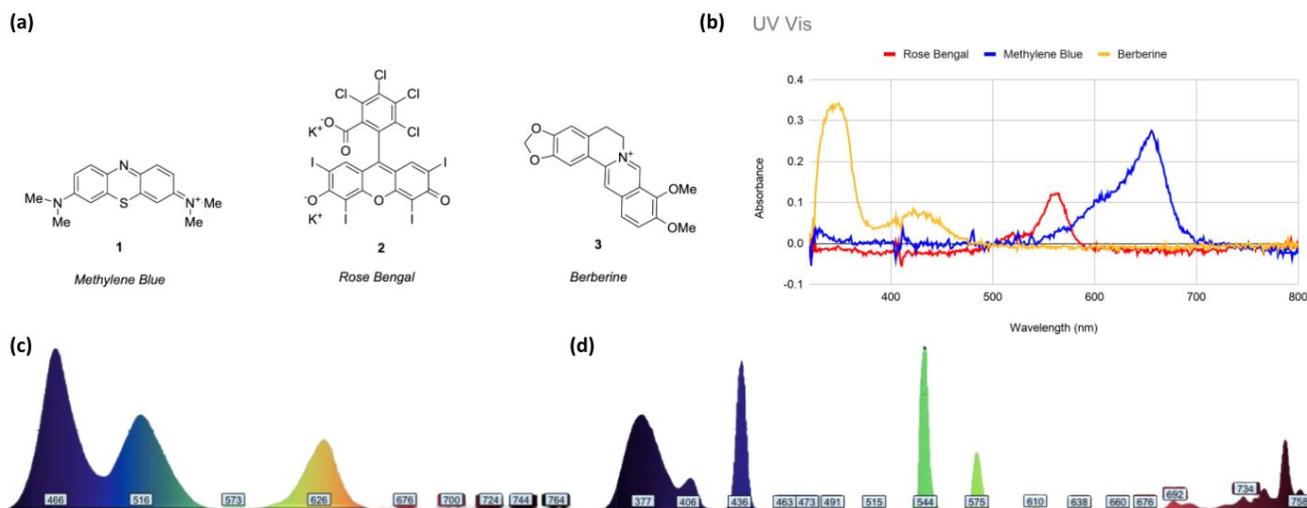
Such medicinal properties stem from its ability to intercalate and incur oxidative damage in guanine base pairs in DNA (4-8). Here, we sought to spectroscopically quantify berberine's efficiency as a photosensitizer in singlet oxygen production with two commercially available photosensitizer dyes (11, 12, 13).



**Figure 1** (a) Berberine intercalates with DNA and incurs oxidative damage on guanine base pairs, ultimately leading to cell death. Irradiation of the berberine-DNA complex allows for the generation of singlet oxygen, which causes DNA cleavage at guanine residues. (b) Experimental design & photoirradiation of NMR tubes. NMR tubes were flushed with oxygen and then irradiated for 60-minute intervals while monitored with  $^1\text{H}$  NMR in between each interval of irradiation. Such an experimental set up allowed for the tracking of endoperoxide formation in alpha terpinene over time, allowing us to view our tested singlet oxygen photosensitizers' efficiency. (c) Molecular orbital diagram of various oxygen species. The unique pairing of electrons in singlet oxygen's pi antibonding 2p orbital accounts for its high reactivity, and its medicinal potential. (d) Jablonski Diagram depicting the electronic transitions that occur as a photosensitizer excites oxygen into singlet oxygen. As photosensitizers become excited and undergo a phosphorescence down from their triplet excited state to their

singlet ground state, energy is transferred to triplet oxygen, which then becomes excited into its reactive singlet form. (e) From left to right: NMR tubes for photochemistry, including control, rose bengal, berberine, and methylene blue.

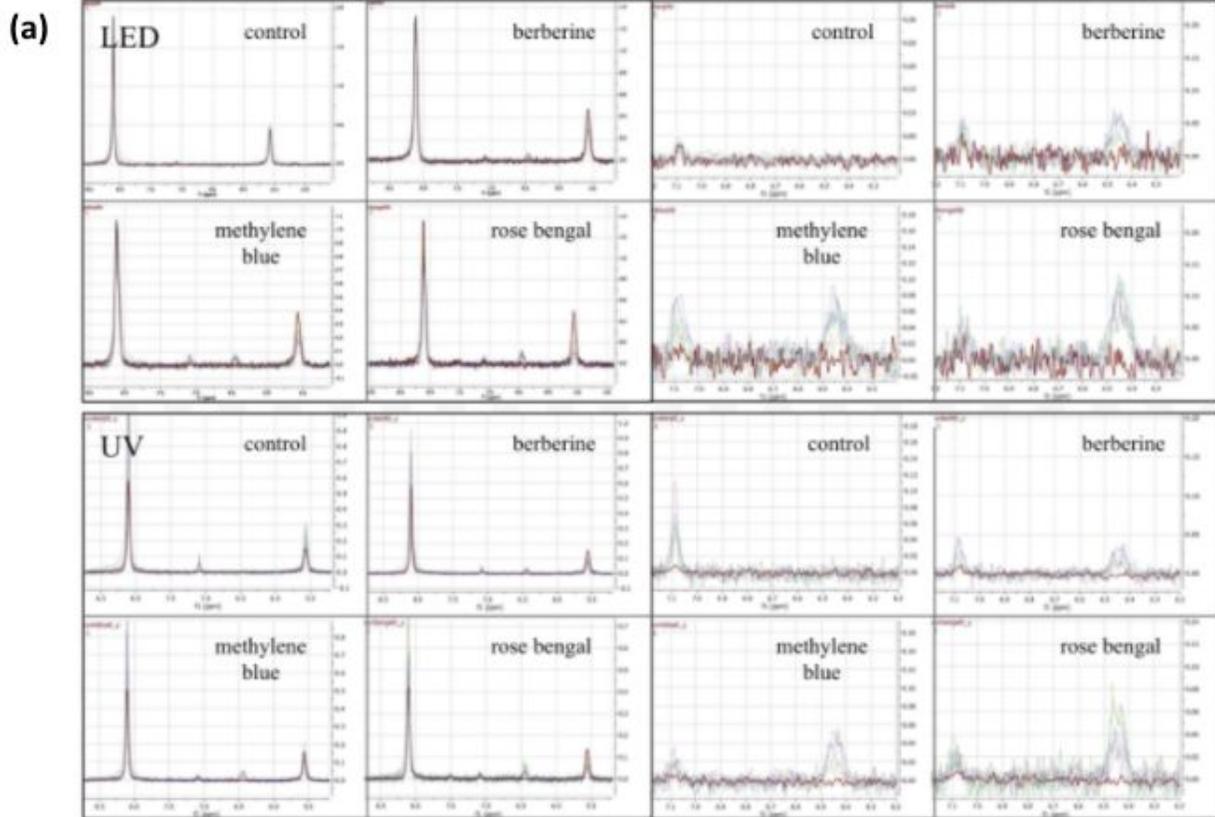
Upon irradiation of the berberine-DNA complex, berberine is able to photosensitize triplet oxygen to singlet oxygen (9, 10). Singlet oxygen is a reactive oxygen species that derives its reactivity from its electron pairing. It differs from triplet oxygen in its pairing of electrons in its pi antibonding 2p orbital (Fig. 1c) (19). The reactivity of the singlet oxygen generated results in specific oxidation of guanine base pairs. This is the putative mechanism for berberine's biological activity, and we have previously reported photochemically activated antimicrobial activity of berberine and its analogs (33).



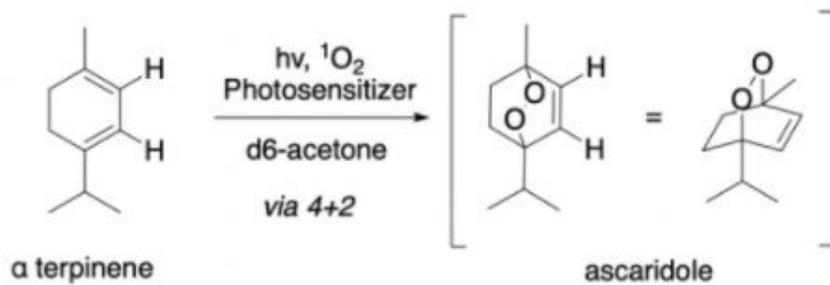
**Figure 2** (a) Chemical structures of three photosensitizers utilized in this comparative study. All photosensitizers are commercially available, and are proven to generate singlet oxygen upon their photoirradiation as a result of the transference of energy during phosphorescence from an excited state to a ground state. (b) Ultraviolet-visible spectroscopy of rose bengal, methylene blue and berberine. The varying absorbances of tested photosensitizers provided a need for varying light sources to ensure irradiation sources overlapped with the excitation range of tested photosensitizers. (c) Emission spectra of LED irradiation source. (d) Emission spectra of UV irradiation source. Both UV and LED irradiation sources were used to maximize overlap with photosensitizer excitation ranges.

Singlet oxygen production can be spectroscopically monitored by trapping the reactive oxygen species with a diene via a 4+2 Diels Alder-like cycloaddition reaction (Fig. 3b) and quantified by determining the resulting concentration of the endoperoxide as well as the

concentration of the cymene, a product resulting from the degradation of the endoperoxide as well as from the generation of a CH radical on alpha terpinene (Fig 3c) (32, 36). Here, we used alpha terpinene, a commercially available natural product terpene with a cyclic 1,3-diene to trap singlet oxygen generated by excitation of each photosensitizer. The reactions were monitored by  $^1\text{H}$  nuclear magnetic resonance (NMR) spectroscopy (Fig. 3a). This has been previously reported as a method of singlet oxygen determination (26) but an extensive study of this applied to berberine in comparison to other commercially available photosensitizers has not been previously reported. Additionally, time dependent density functional theory (TD-DFT) was used to model excited state frontier molecular orbitals of the photosensitizers used in this study.



(b)



(c)

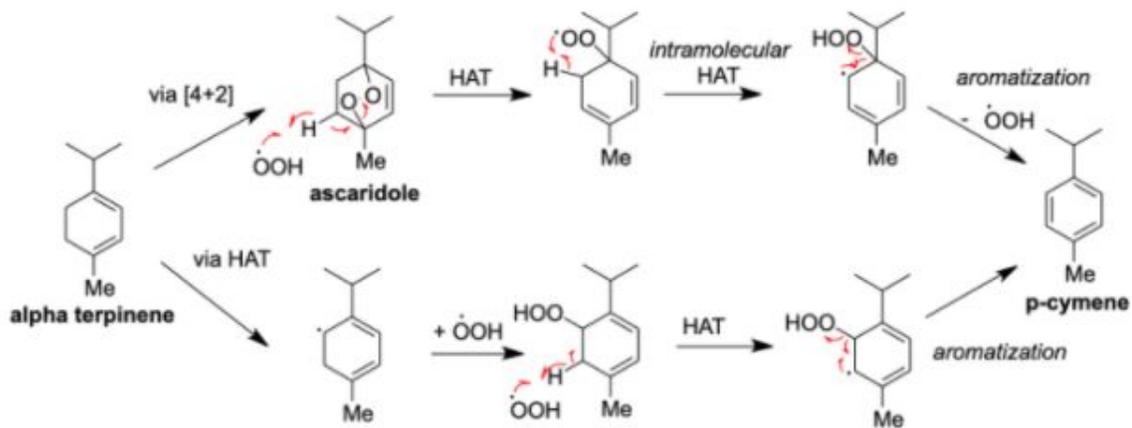


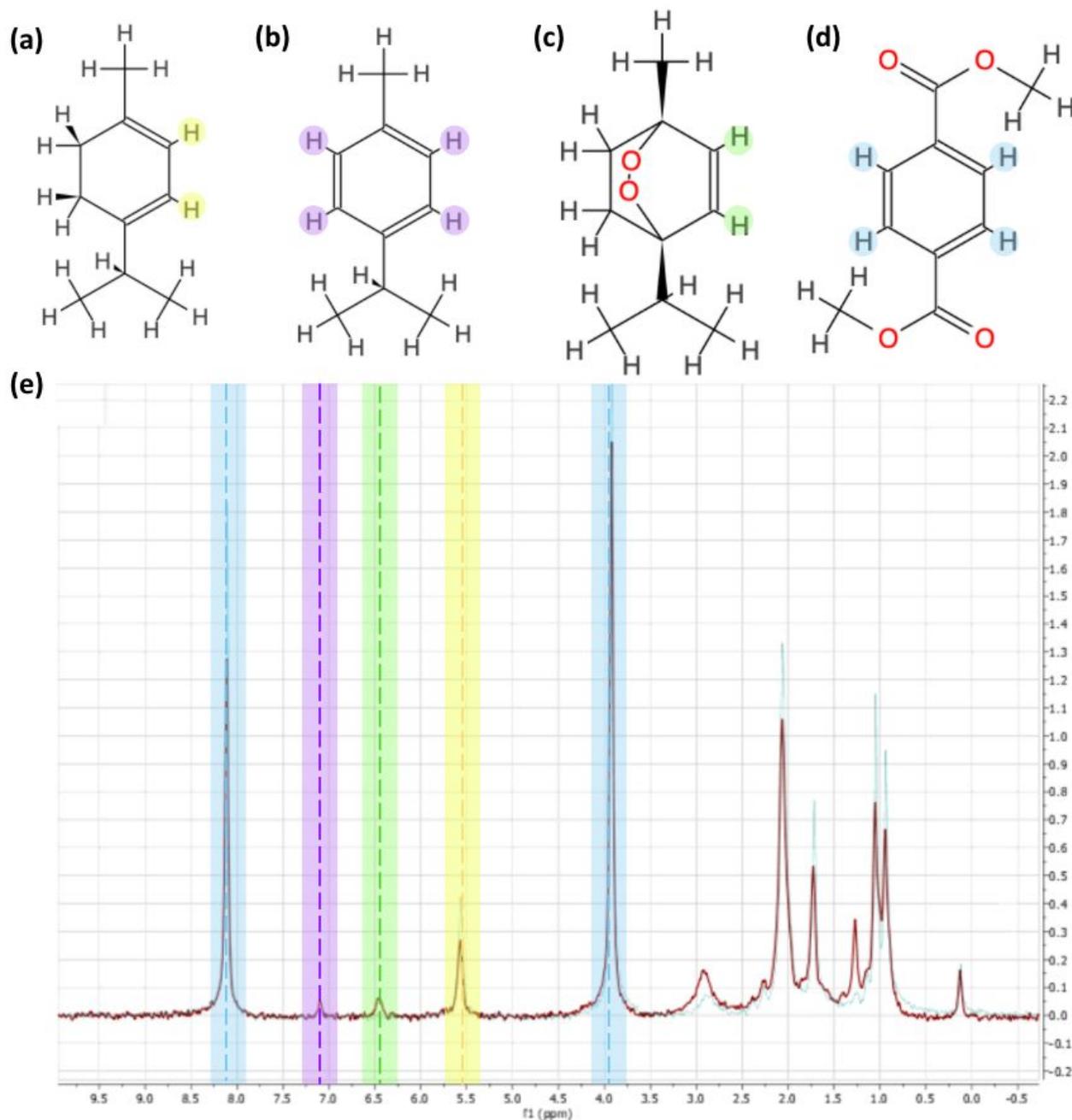
Figure 3. (a) Reaction schematic depicting Diels Alder 4+2

like reaction between singlet oxygen and alpha terpinene.

The diene of the alpha terpinene reacts with the singlet oxygen to trap it into an endoperoxide, which can be monitored using NMR and quantified by integrating endoperoxide and cymene peaks. (b)  $^1\text{H}$  nuclear magnetic resonance tracking of endoperoxide and cymene formation of all three photosensitizers used in this study after 60-minute intervals over five hours of irradiation. Peaks at 6.44 represent the appearance of an endoperoxide, and peaks at 7.13 represent the appearance of cymene over time as photosensitizers within NMR tubes were irradiated using various light sources. (c) Potential mechanisms for the oxidation of alpha terpinene to ascaridole and cymene, with ascaridole being the major product and cymene being the minor product. Integration of both on NMR spectra was used to quantify singlet oxygen production.

## Results

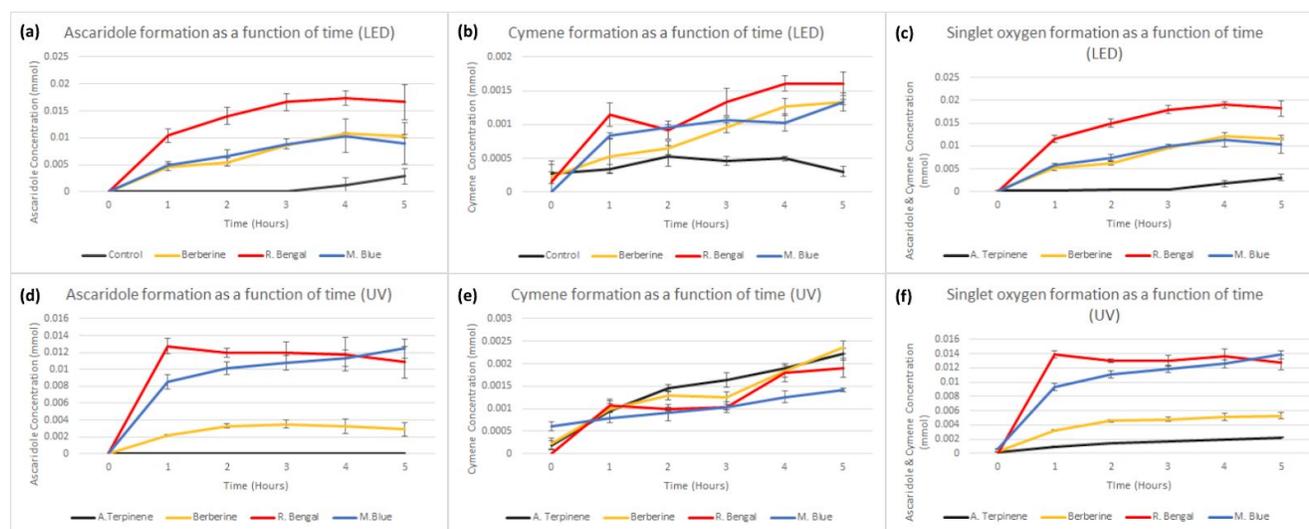
All three photosensitizers were irradiated with both LED and UV light with the following emission spectrums in order to ensure that radiation occurred within their respective excitation ranges.  $^1\text{H}$  nuclear magnetic resonance (NMR) spectroscopy was used to quantify singlet oxygen production as a function of time, taken in 60-minute intervals.



**Figure 4.** Singlet oxygen production was quantified through the integration of a doublet with a chemical shift of 6.44 ppm and doublet of doublets with a chemical shift of 7.13 ppm. The aromatic protons of cymene (b), the minor oxidative product of alpha terpinene generated a doublet of doublets at 7.13 ppm. In comparison, the vinyl protons of ascaridole (c) generated a doublet at 6.44 ppm. Vinyl protons of alpha terpinene (a) generated a doublet at 5.5 ppm, and dimethyl terephthalate's aromatic protons (d) generated singlets at about 8.1 ppm and 4.0 ppm.

Over time, endoperoxide formation was revealed through the appearance of a doublet with a chemical

shift of 6.44 ppm, while cymene formation was revealed through the appearance of a doublet of doublets with a chemical shift of 7.13 ppm. The chemical shift of this doublet coincides with literature precedent of <sup>1</sup>H spectra of ascaridole, hence, we were able to use this doublet as a way of quantifying endoperoxide formation (27). The integral of this doublet was used to quantify endoperoxide formation over time in both studies, and the integral of the doublet of doublets was used to quantify cymene formation over time in both studies. The sum of both the micromolar concentration of endoperoxide and cymene was used to yield (Fig. 5c, 5f



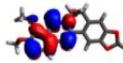
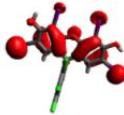
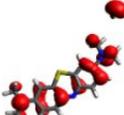
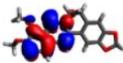
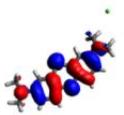
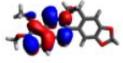
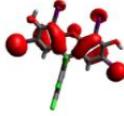
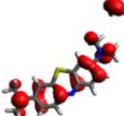
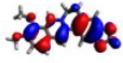
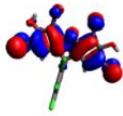
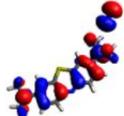
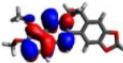
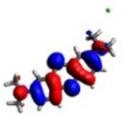
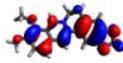
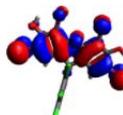
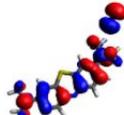
**Figure 5.** (a) Ascaridole formation as a function of time was quantified for groups irradiated under LED light through the integration of the peak with a chemical shift of 6.44 ppm on NMR <sup>1</sup>H spectra on MestReNova for 60 minute intervals. The formula  $\frac{Ccal \cdot I_x \cdot Ncal}{Ical \cdot N_x}$  was used to find the molar concentration of the endoperoxide, where C, I, and N stand for concentration, integral, and nuclei of the peak respectively, and cal and x refer to the calibrant and target compound respectively. A positive correlation between time and ascaridole formation was found under irradiation by LED light, and seemed to be dependent on photosensitizers due to the lack of significant ascaridole formation in the control group. (b) Ascaridole formation as a function of time under UV light was quantified similarly as described in a. Ascaridole formation was also found to have a positive correlation with time spent irradiating under UV light, and seemed to be dependent on photosensitizers, as shown through the lack of ascaridole production in the control group. (c) Cymene formation as a function of time under LED light was quantified similarly as described in a. Cymene formation was also found to have a positive correlation with time spent irradiating under LED light, and seemed to be dependent on photosensitizers, as shown through the disparities of cymene production between the photosensitizers. (d) Cymene formation as a function of time under UV light was quantified similarly as described in a. Cymene formation was also found to have a positive correlation with time spent irradiating under UV light, but did not seem to be dependent on photosensitizer, as all photosensitizers performed similarly, including the control

group. We believe this may be due to UV's role in radical formation and substitution. (e) Singlet oxygen formation as a function of time was quantified for groups irradiated under LED light through summing the molar concentrations of the endoperoxide and cymene formation under LED light. Singlet oxygen formation was found to have a positive correlation with time spent irradiating under LED light. (f) Singlet oxygen formation as a function of time was quantified for groups irradiated under UV light through the sums of the molar concentrations of the endoperoxide and cymene formation under UV light. Singlet oxygen formation was also found to have a positive correlation with time spent irradiating under UV light.

Analysis of <sup>1</sup>H NMR spectra revealed that, when irradiated with white LED lights, endoperoxide and cymene formation was most efficient with rose bengal and lowest in the presence of berberine and methylene blue, which performed similarly. This suggests that berberine and methylene blue have lower photosensitizing properties in comparison to rose bengal. Berberine and methylene blue's endoperoxide formation over time remained relatively similar under both LED and UV irradiation conditions, whereas rose bengal had significantly worse endoperoxide formation under UV irradiation. Such can be attributed to the imperfect overlap of the emission spectra of the

irradiation sources and absorbance of the photosensitizers, regardless of whether LED or UV light was used. Under UV conditions, however, photosensitizer appeared to have no influence on cymene production, which we hypothesize is due to UV light's ability to generate and substitute the CH radical.

As expected, no endoperoxide was detected in the control tubes without any photosensitizer added. Statistical analysis using two-tailed T-testing determined that results were statistically significant to a 95% confidence level.

	Berberine	Rose Bengal	Methylene Blue		Berberine	Rose Bengal	Methylene Blue
LUMO + 1 (singlet)	eV = -2.11 	eV = -1.825 	eV = -1.448 	LUMO (triplet)	eV = -3.999 	eV = -3.018 	eV = -5.048 
LUMO + 1 (triplet)	eV = -2.226 	eV = -2.336 	eV = -1.463 	HOMO (singlet)	eV = -5.931 	eV = -6.360 	eV = -6.141 
LUMO (singlet)	eV = -2.777 	eV = -2.414 	eV = -3.970 	HOMO (triplet)	eV = -6.346 	eV = -6.534 	eV = -6.247 

**Figure 6.** Excited state molecular orbitals of photosensitizers are visualized with Avogadro after being calculated by ORCA time dependent density functional theory (TD-DFT). Molecular orbitals in red represent positive overlap, while molecular orbitals in blue represent negative overlap.

Computationally, all photosensitizers had enough energy from the gap between triplet excited and singlet ground state to generate singlet oxygen, which is 0.98 eV. (9) Lifetimes of excited states and kinetics of excited state decay can be extrapolated from this data using Fermi's Golden Rule; however, that is beyond the scope of our current research (28).

## Materials and Methods

**Photochemistry** Experimental NMR tubes were prepared with photosensitizer (10 mol%), dimethyl terephthalate (1 eq, 8.9 mg), alpha terpinene (1 eq, 6.25 mg), and d6-acetone (0.6 mL). Deuterated acetone was chosen due to the prolonged lifetime of singlet oxygen (529 $\mu$ s), as previously reported in the literature (35). A set of control tubes were prepared with dimethyl terephthalate (1 eq, 8.9 mg), alpha terpinene (1 eq, 6.25 mg), and d6-acetone (0.6 mL) without any photosensitizer, and these were likewise subjected to the same photoirradiation conditions. The headspaces of the NMR tubes were purged with oxygen (Boost Oxygen, >95%).

**Physical Methods** Emission spectra of LED lights and UV lights used in this study were collected on an Ocean Optics spectrometer. UV-visible spectra were collected in acetone in a BioRad SmartSpec 3000 UV-visible spectrophotometer or a Thermo Electron Genesys 5 UV-visible spectrophotometer in quartz cuvettes. Tubes were photo irradiated in 60-minute intervals with LED and UV light, respectively. 1H NMR spectra were collected on a Nanalysis NMRready 60PRO 60 MHz benchtop spectrometer in d6-acetone.

Endoperoxide formation was quantified by 1H nuclear magnetic resonance at 60-minute intervals for 5 hours by integration of the endoperoxide peak at 6.44 ppm (d, J = 2.28 Hz). Cymene formation was quantified by 1H nuclear magnetic resonance at 60-minute intervals for 5 hours by integration of the cymene peak at 7.13 ppm (dd, J = 3.6, 2.4 Hz). Dimethyl terephthalate was used as an internal standard, and was chosen because its signature resonances (8.1 ppm, Ar-H; 3.95 ppm, -OCH3) did not overlap with signals from alpha terpinene or ascaridole. NMR spectra were processed and visualized on the MestreNova software package (34).

**Chemicals** Photosensitizers tested include berberine (MaxSun, >97%), rose bengal (HiMedia Laboratories, > 98%), and methylene blue (HiMedia Laboratories, > 98%), and were used without further purification.

Deuterated acetone was purchased from Martek Isotopes (>99% acetone-d<sub>6</sub>, >99.8% deuterated) and was used without further purification. Dimethyl terephthalate (>95%) and alpha terpinene (> 95%, by GC) were purchased from AK Scientific and used without further purification.

**Statistical Analysis** P-values were calculated using the following equation:  $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2(\frac{1}{n_1} + \frac{1}{n_2})}}$ , where “t”, also known as the t-value, is the quotient of the difference between two means, denoted by  $\bar{x}$ , divided by the square root of the times sample variance squared, denoted by  $s^2 = \frac{\sum_{i=1}^{n_1}(x_i - \bar{x}_1)^2 + \sum_{i=1}^{n_2}(x_i - \bar{x}_2)^2}{n_1 + n_2 - 2}$ , multiplied by the difference of the reciprocal of the two populations, referred to by the variable n. After calculating the t-value, the value was compared to the t-distribution critical value table to derive the corresponding critical value at a 95% confidence level. If the t-value calculated was less than the critical value, the p-value will be less than 0.05, which signifies a statistically significant value at a 95% confidence level.

**Computational Analysis** All photosensitizers were geometrically optimized by density functional theory using ORCA, an open source, ab initio quantum mechanical modeling package (29) and visualized using Avogadro, an open source molecular modeling software (30). Calculations were performed with the B3LYP functional, def2-SVP basis set, and with the CPCM implicit solvation model using the dielectric constant of deuterated acetone. Computational analysis of excited state electronic structures was performed with time-dependent density functional theory (TD-DFT) (31) using the B3LYP functional, def2-TZVP basis set, and a RijCosX approximation on ORCA. All calculations were performed on a Dell PowerEdge 710 server with a 24 core Intel Xeon X5660 processor @ 2.80GHz and 32GB RAM.

## Discussion

In situ quantification of singlet oxygen production is essential in probing the efficiency of photosensitizers that have potential use in photodynamic therapy. Here, the photosensitizing efficiency of berberine, a natural product alkaloid, was monitored using NMR and was spectroscopically quantified via trapping singlet oxygen with alpha terpinene, a natural product 1,3-diene, as ascaridole, its endoperoxide derivative. As expected, all three compounds demonstrated photosensitizing capabilities, and this is corroborated with our TD-DFT calculations on excited state molecular orbital energies. Two-tailed statistical analysis on endoperoxide concentrations revealed that all are photosensitizers in LED and UV light besides methylene blue and berberine for certain time periods, wherein methylene blue was not significant against the control for time period 5 under LED light and berberine was not significant against the control for time period four under

UV light. Against berberine, methylene blue was not statistically different for time periods 4 & 5 under LED light and time periods 1 & 4 under UV light, and rose bengal was not statistically different for time period 5 under LED light and time period 4 under UV light. Endoperoxide concentration was used to evaluate statistical significance due to UV light's ability to generate and substitute radicals, which could lead to the production of cymene in the absence of singlet oxygen. While, in concordance with literature precedent, the appearance of the 4+2 endoperoxide product observed by 1H NMR indicated that berberine is indeed a singlet oxygen photosensitizer, this work also demonstrates that, contrary to our initial hypothesis, berberine is a less efficient photosensitizer than other commercially available photosensitizer dyes, including rose bengal, when irradiated with either UV-C light or visible light.

Under UV irradiation, berberine performed similarly well to methylene blue in singlet oxygen photosensitizing efficiency. This was surprising, especially given how berberine tends to absorb within the UV range. Furthermore, berberine tended to aggregate over time as evidenced by the appearance of yellow granules, whereas methylene blue did not, possibly resulting in reduced performance; however, both berberine and methylene blue demonstrated similar performance in their photosensitizing abilities.

The quantification of berberine's photosensitizing abilities can serve as a foundation for semi-synthetic berberine analogs to optimize its efficacy as a singlet oxygen photosensitizer. The study design described here can be applied to other singlet oxygen photosensitizers to quantify their proficiency in generating reactive oxygen species, which can also be used comparatively with their analogs. The quantification of singlet oxygen production can direct the future synthesis of berberine analogs, and identify medicinally significant singlet oxygen photosensitizers.

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All authors have given approval to the final version of the manuscript.

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## ABBREVIATIONS

ATerp, Alpha Terpinene; Berb, Berberine; RBengal, Rose Bengal; MBlue, Methylene Blue; UV, Ultraviolet; TDDFT, Time-Dependent Density Functional Theory; DFT, Density Functional Theory; NMR, Nuclear Magnetic Resonance; DNA, Deoxyribose Nucleic Acid; ppm, parts per million; LED, Light Emitting Diode;

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