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Article

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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SUMMARY

Berberine, a natural product alkaloid, has been shown to exert biological activity via in situ production of singlet oxygen 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, thereby halting cell replication and 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 postulated that the singlet oxygen produced by photoirradiation of berberine would be superior in terms of singlet oxygen production 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 1H 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 of 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. Berberine's use has been documented in ancient medicine as early as 3000 B.C. to treat various diseases such as diabetes, infections, and inflammation (1). It 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) Such oxidative damage in DNA can be utilized in many medicinal settings as such damage incurs cell death. Here, we sought to spectroscopically quantify berberine's efficiency as a photosensitizer in singlet oxygen production with two commercially available photosensitizer dyes (9–11).

Singlet oxygen formation occurs when singlet oxygen photosensitizers are excited by absorbing a photon of light with the appropriate quantum energy and returning to their ground state (12). This occurs when the initial absorbance of a photon is excited from the ground state to the second excited state and then undergoes an internal conversion back to its initial excited state (Figure 1a). The intersystem crossing from singlet excited state to triplet excited state, wherein berberine's electronic state is excited to the LUMO+1 shell, allows for a relaxation back down from triplet excited state to singlet ground state, which can then transfer energy to the ground state triplet oxygen, exciting it to the singlet oxygen state (Figure 1a).

Upon irradiation of the berberine-DNA complex, berberine can photosensitize triplet oxygen to singlet oxygen (13, 14). 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 (Figure 1b) (15). The reactivity of the singlet oxygen generated allows for the 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

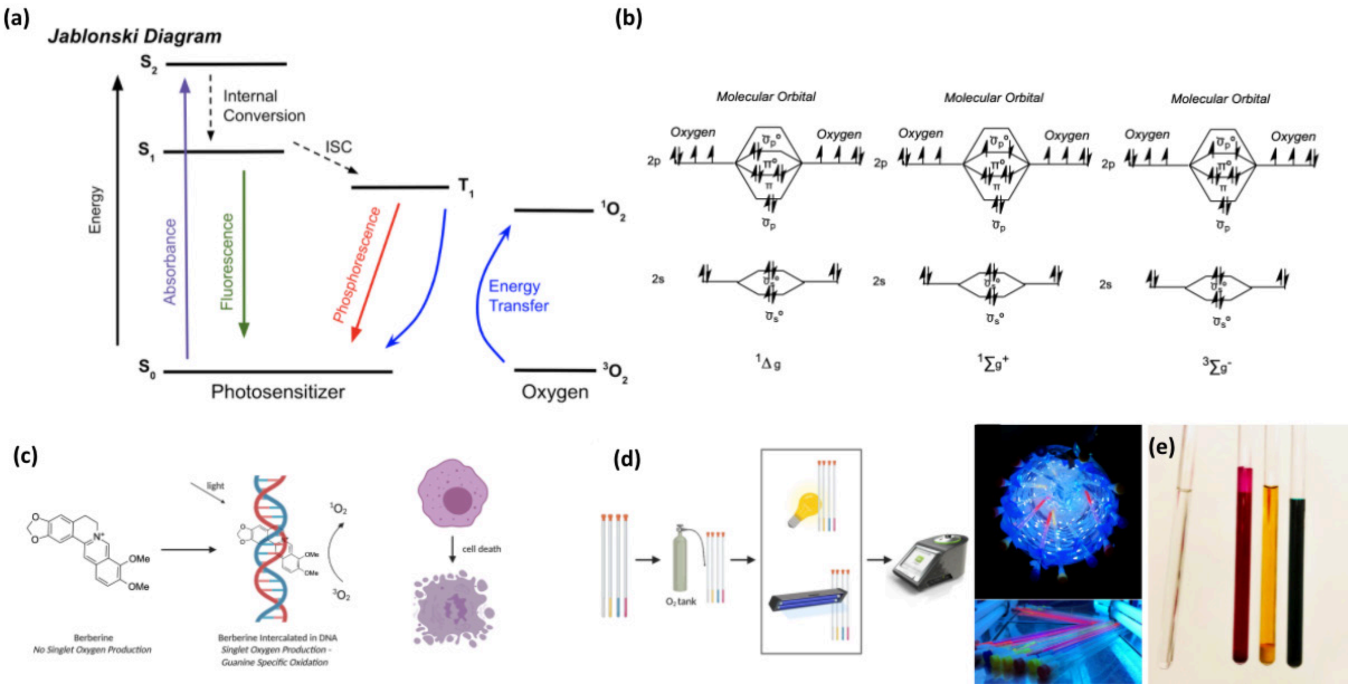


Figure 1: Berberine's Mechanism of Action and Experimental Setup. (a) Jablonski Diagram depicting the electronic transitions that occur as a photosensitizer excites oxygen into singlet oxygen. As photosensitizers become excited and undergo a phosphorescence down from their triplet excited state to their singlet ground state, energy is transferred to triplet oxygen, which then becomes excited into its reactive singlet form. **(b)** 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. **(c)** 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. **(d)** Experimental design & photoirradiation of NMR tubes. NMR tubes were flushed with oxygen and then irradiated for 60-minute intervals while monitored with 1H 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. **(e)** From left to right: NMR tubes for photochemistry including control, rose bengal, berberine, and methylene blue.

(Figure 1c) (16).

In photodynamic therapy, visible light and the active drug (photosensitizer) are combined, resulting in the activation of cytotoxic agents that kill tumor cells (17, 18). Controlling the production of singlet oxygen by photosensitizers has been utilized to destroy cancer cells, pathogenic microbes, and unwanted tissue (19). Commercially available photosensitizers such as methylene blue and rose bengal have been studied for their potential in photodynamic therapy of cancer through their production of singlet oxygen (Figure 2a) (20, 21). Previous studies have indicated that rose bengal is a more efficient photosensitizer than methylene blue in photodynamic therapy; however, studies comparing photosensitizing abilities across all three photosensitizers, berberine, methylene blue and rose bengal, have yet to be done (22). Berberine's extensive literature on its singlet oxygen capabilities as well as its high UV absorbance suggest that it is a capable singlet oxygen photosensitizer (7). We postulated that the singlet oxygen produced by photoirradiation of berberine would be superior in its photosensitizing efficacy to the other photosensitizers when irradiated with UV light, but inferior under visible light conditions due to its strong absorbance of UV wavelengths.

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 (Figure 3a). This reaction can be 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 (Figure 3b) (23, 24). 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 (Figure 1d–e). The reactions were monitored by 1H nuclear magnetic resonance (NMR) spectroscopy. This has been previously reported as a method of singlet oxygen determination (25), 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. Our study found rose bengal was superior to both methylene blue and rose bengal in singlet oxygen production and berberine and methylene blue performed similarly.

RESULTS

All three photosensitizers were irradiated with both LED and UV light to ensure that radiation occurred within their respective excitation ranges (Figure 2b–2c). 1H nuclear magnetic resonance (NMR) spectroscopy was used to quantify singlet oxygen production as a function of time, taken in 60-minute intervals. 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.

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 (Figure 4a–4b). The chemical shift of the doublet at 6.44 ppm coincides with literature precedent of 1H spectra of ascaridole; hence, we were able to use this doublet as a way of quantifying endoperoxide formation (26). The peaks with chemical shifts of 4.0 ppm and 8.2 ppm correspond to the protons of dimethyl terephthalate and alpha terpinene, respectively, which were utilized to quantify the amounts of endoperoxide and cymene formation through integration (Figure 4c–4e). The integral of this doublet was used to quantify endoperoxide formation over time in both UV and LED studies, and the integral of the doublet of doublets was used to quantify cymene formation over time in both aforementioned studies (Figure 4f). The sum of both the micromolar concentration of endoperoxide and cymene was used to yield singlet oxygen formation as a function of time under both LED and UV irradiation conditions (Figure 5c, 5f).

Analysis of 1H NMR spectra revealed that, when irradiated with white LED lights, endoperoxide and cymene formation was most efficient with rose bengal and lower in the presence of berberine and methylene blue, which performed similarly (Figure 5b). This suggests that berberine and methylene blue have lower photosensitizing properties in comparison to rose bengal (Figure 4d). Berberine and methylene blue's endoperoxide formation over time remained relatively similar under both LED and UV irradiation conditions (Figure 5a, 5d). Discrepancies in the formation of the endoperoxide, where berberine performed more poorly, could be attributed to its aggregation over time, in which a yellow precipitate could be observed (Figure 1e). Rose bengal had notably worse endoperoxide formation under UV irradiation (Figure 5d). Berberine's poor photosensitizing efficacy 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 type appeared to have no influence on cymene production, which we presume is due to UV light's ability to generate and substitute the CH radical

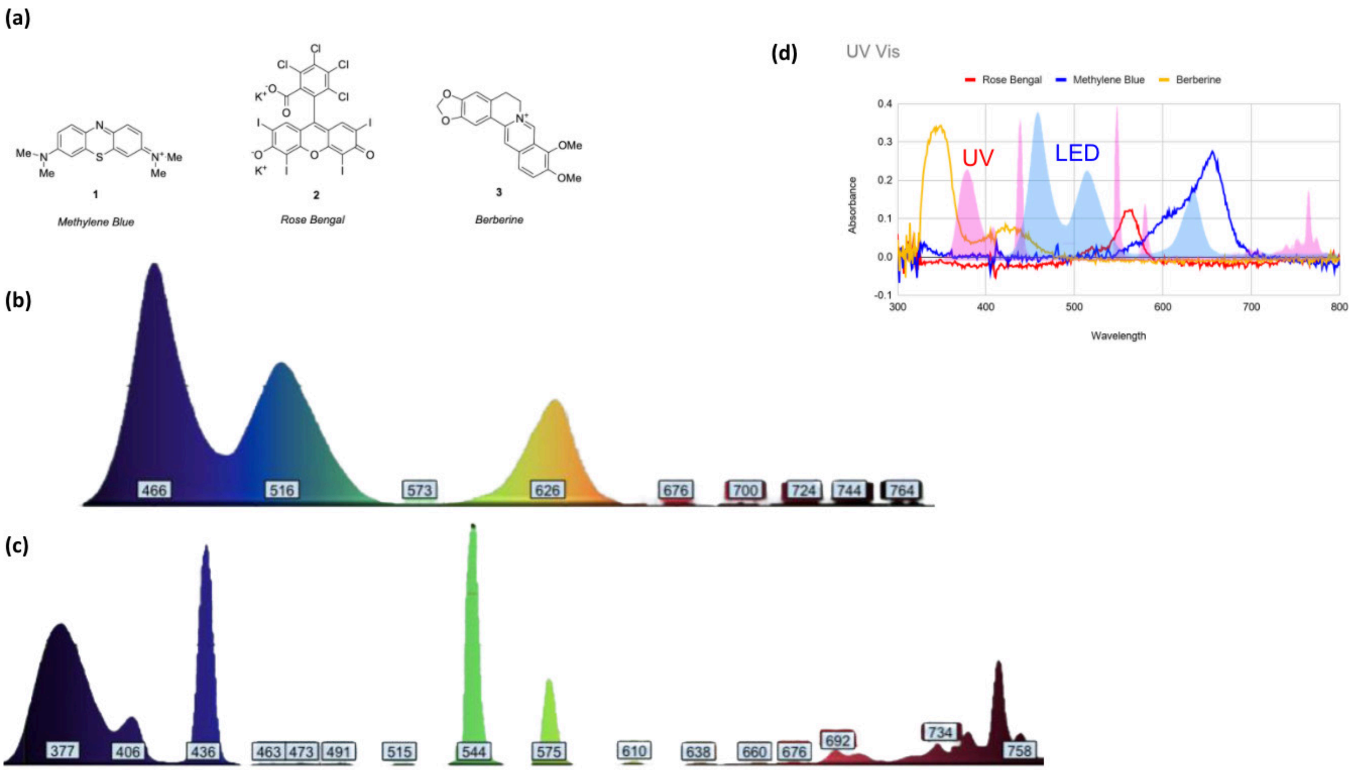


Figure 2: Absorption Spectra of Tested Commercially Available Photosensitizers. (a) Chemical structures of three photosensitizers utilized in this comparative study. All photosensitizers are commercially available and are proven to generate singlet oxygen upon their photoirradiation as a result of the transference of energy during phosphorescence from an excited state to a ground state. **(b)** Emission spectra of LED irradiation source. **(c)** Emission spectra of UV irradiation source. Both UV and LED irradiation sources were used to maximize overlap with photosensitizer excitation ranges. **(d)** 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.

(Figure 3b, 5e). As expected, no endoperoxide was detected in the control tubes without any photosensitizer added (Figure 5a, 5d). A two-tailed t-test was used to determine whether or not each molecule (berberine, rose bengal, and methylene blue) was able to act as a photosensitizer. The two-tailed t-test was used to verify that at each time period the three proposed molecules were able to act as a photosensitizers when comparing them to a control group which was a vial that contained no photosensitizer (therefore just deuterated acetone, dimethyl terephthalate, and alpha terpinene). Significance was found when comparing ascaridole production of those photosensitizers versus the production of ascaridole of the control groups at each respective time intervals to which they were statistically significant with a p-value < 0.05 (other than methylene blue at time period five, which had a p value of 0.11). That is, all tested photosensitizers were indeed photosensitizers.

Two-tailed statistical analysis on endoperoxide concentrations revealed that all compounds tested are indeed 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 five (hour five) under LED light and berberine was not significant against the control for time period four under UV light. Against

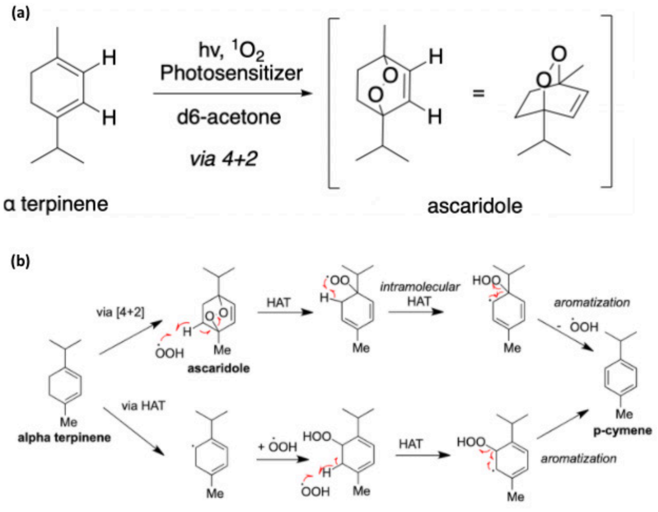


Figure 3: Mechanism behind Experimental Setup. (a) Reaction schematic depicting Diels Alder 4+2-like reaction between singlet oxygen and alpha terpinene. The diene of the alpha terpinene reacts with the singlet oxygen to trap it into an endoperoxide, which can be monitored using NMR and quantified by integrating endoperoxide and cymene peaks. **(b)** 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 doublets signifying ascaridole and cymene products on NMR spectra was used to quantify singlet oxygen production.

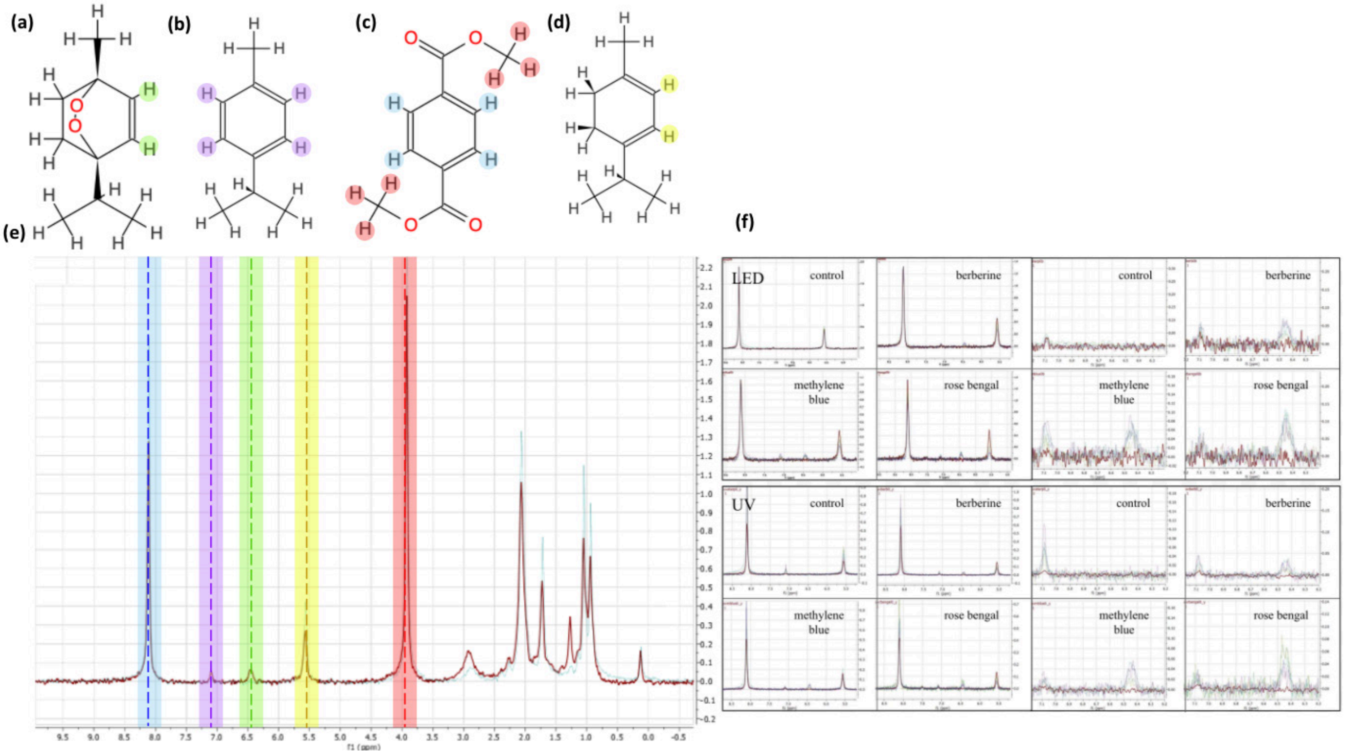


Figure 4: Tested Photosensitizers and their Chemical Shifts on NMR. Singlet oxygen production was quantified through the integration of a doublet with a chemical shift of 6.44 ppm and doublet of doublets with a chemical shift of 7.13 ppm **(e)**. The aromatic protons of cymene **(b)**, the minor oxidative product of alpha terpinene, generated a doublet of doublets at 7.13 ppm. In comparison, the vinyl protons of ascaridole **(a)** generated a doublet at 6.44 ppm. Vinyl protons of alpha terpinene **(d)** generated a doublet at 5.5 ppm, and dimethyl terepthalate's aromatic protons **(c)** generated a doublet of doublets at about 8.1 ppm while its methoxys generated a singlet at about 4.0 ppm. **(f)** 1H 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.

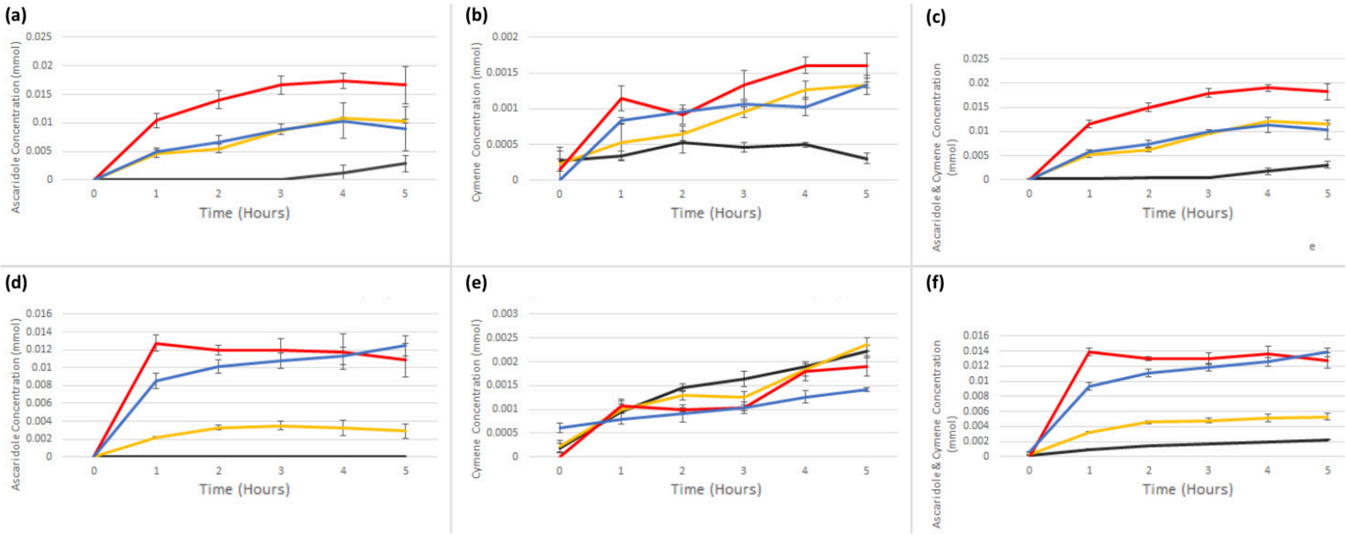


Figure 5: Experimental Data of Singlet Oxygen Formation as a Function of Time. The formation of ascaridole, cymene, and singlet oxygen were all quantified via 1H NMR. Results for controls are noted in black, berberine in yellow, rose bengal in red, and methylene blue in blue. **(a)** Ascaridole formation as a function of time was quantified for groups irradiated under LED light. 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 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 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 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.

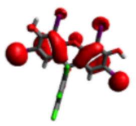
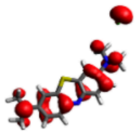
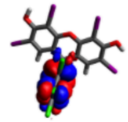
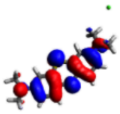
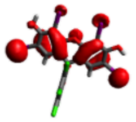
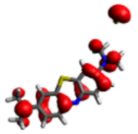
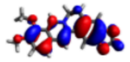
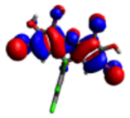
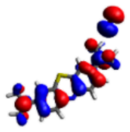
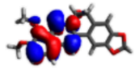
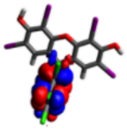
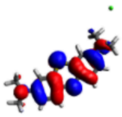
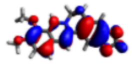
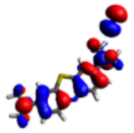
	Berberine	Rose Bengal	Methylene Blue		Berberine	Rose Bengal	Methylene Blue
LUMO + 1 (singlet)	eV = -2.11 	eV = -1.825 	eV = -1.448 	LUMO (triplet)	eV = -3.999 	eV = -3.018 	eV = -5.048 
LUMO + 1 (triplet)	eV = -2.226 	eV = -2.336 	eV = -1.463 	HOMO (singlet)	eV = -5.931 	eV = -6.360 	eV = -6.141 
LUMO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

Table 1: Computational Modeling of Tested Photosensitizers. 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.

berberine, methylene blue was not statistically different for time periods four & five (hour four and five) under LED light and time periods one & four (hour one and four) under UV light, and rose bengal was not statistically different for time period five under LED light and time period four (hour four) under UV light. Cymene statistical testing proved cymene production to be largely statistically insignificant, which we believe to be a result of the fact that UV light itself can catalyze the generation of cymene.

Computationally, all photosensitizers had enough energy from the gap between triplet excited and singlet ground state, which is 0.98 eV, to generate singlet oxygen (Table 1) (27). Based on the difference between the triplet excited and singlet ground state energies of each of the photosensitizers, gap energies can be calculated, wherein rose bengal had the highest gap energy of 3.342 eV, berberine had a gap energy of 1.932 eV, and methylene blue had a gap energy of 1.093 eV. Such gap energies correspond to the photosensitizers' ability to excite triplet oxygen to singlet oxygen and reflect the physical results obtained from our assay wherein rose bengal and berberine tended to be the best photosensitizers. Lifetimes of excited states and kinetics of excited state decay can be extrapolated from this data using Fermi's Golden Rule (28); however, that is beyond the scope of our current research.

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, which were corroborated with our TD-DFT calculations on excited state molecular orbital energies. 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 conjecture, 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 given that berberine tends to absorb within the UV range (Figure 2d). Furthermore, berberine tended to aggregate over time as evidenced by the appearance of yellow granules (Figure 1e), whereas methylene blue did not, possibly resulting in reduced performance; however, both berberine and methylene blue demonstrated similar performance in their photosensitizing abilities. Although berberine did perform significantly better than expected, berberine is not a particularly strong photosensitizer. The strongest absorption peak of berberine under ultraviolet-visible spectroscopy falls roughly at 325 nm (Figure 2d) rather than 375 nm as represented in the UV irradiation source emission spectra (Figure 2c). This difference between the absorption peak of berberine and the UV emission spectra may be what caused berberine's weak performance as a photosensitizer (Figure 2d).

Computational analysis of photosensitizers revealed that all photosensitizers were indeed functional photosensitizers, and that rose bengal is a better photosensitizer than berberine and methylene blue, with methylene blue being the worst photosensitizer of the three. However, TD-DFT limitations may occur due to the use of the RijCosX calculations, wherein the CosX grid may cause minor numerical limitations. Furthermore, the use of solvation models may impact the overlap of computational results and observed UV absorption spectra.

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. As stated in the introduction, singlet oxygen photosensitizers are indicated in a vast variety of medicinal applications, such as in the photodynamic treatment of cancer. Identifying medicinally significant singlet oxygen photosensitizers has implications in the treatment of a variety of fatal diseases.

MATERIALS AND METHODS

Chemicals

Photosensitizers tested include commercially available berberine (MaxSun, >97%), rose bengal (HiMedia Laboratories, > 98%), and methylene blue (HiMedia Laboratories, > 98%), which were used as purchased without further purification. Deuterated acetone was purchased from Martek Isotopes (>99% acetone-d6, >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.

Photochemistry

Experimental NMR tubes were prepared with photosensitizer (rose bengal, methylene blue, berberine) (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 as a solvent due to the prolonged lifetime of singlet oxygen (529μs), as previously reported in the literature (29). 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 prior to irradiation and NMR spectroscopy (Boost Oxygen, >95%).

Physical Methods

Emission spectra of LED lights (Minger) and UV lights (MelodySusie) 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 consecutively for 60-minute intervals with LED and UV light, respectively. A 10 minute gap was allowed for 1H NMR spectra to be collected on a Nanalysis NMReady 60PRO 60 MHz benchtop spectrometer in d6-acetone.

Endoperoxide formation was quantified by 1H nuclear magnetic resonance at 60-minute intervals for five 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 five 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 for quantification of endoperoxide formation 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 (30). The formula (Ccal * Ix * Ncal)/(Ical * Nx) 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.

Statistical Analysis

P-values were calculated using the following equation:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2\left(\frac{1}{n_1} - \frac{1}{n_2}\right)}}$$

where t, also known as the t-value, is the quotient of the difference between two means, divided by the square root of the sample variance squared,

$$s^2 = \frac{\sum_{i=1}^{n_1}(x_i - \bar{x}_1)^2 - \sum_{i=1}^{n_2}(x_i - \bar{x}_2)^2}{n_1 + n_2 - 2}$$

multiplied by the difference of the reciprocal of the two population sizes, referred to by the variable n. The calculated t-value was compared to the t-distribution critical value table to derive the corresponding critical value where $p < 0.05$ (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. We used this test to compare photosensitizer (berberine, rose bengal, and methylene blue) versus control (a vial containing deuterated acetone, dimethyl terephthalate, and alpha terpinene) production per time period and irradiation condition for ascaridole and cymene production respectively to test that each tested molecule was indeed a photosensitizer.

Computational Analysis

All photosensitizers were geometrically optimized by density functional theory using ORCA, an open-source, ab initio quantum mechanical modeling package (31) and visualized using Avogadro, an open-source molecular modeling software (32). 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 (31). Computational analysis of excited state electronic structures was performed with TD-DFT (33) 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.

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