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REVIEW ARTICLE

Antimicrobial photodynamic therapy with methylene blue and its derivatives in animal studies: Systematic review

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Abstract

Background: Infections are complications in the wound healing process, and their treatment can lead to antibiotic overuse and bacterial resistance. Antimicrobial photodynamic therapy (aPDT) is used to treat infectious diseases caused by fungi, viruses, or bacteria. Methylene blue (MB) and its derivatives are commonly used dyes in antimicrobial photodynamic therapy (aPDT–MB).

Methods: This study is a PRISMA systematic review of animal models used to discuss the usefulness and therapeutic parameters of aPDT–MB or its derivatives for treating infected skin wounds.

Results: After an extensive literature review, 13 controlled trials totaling 261 animals were selected to evaluate skin infection by leishmaniasis and cutaneous bacterial and fungal infections. All studies found results favoring the use of aPDT–MB. Great variability in parameters was found for radiant exposure from 12 to 360 J/cm^2, MB diluted in saline solution or distilled water, irradiation time from 40 to 3600 s, irradiance most commonly at a maximum of 100 mW/cm^2, and wavelength used mainly in the 630–670 nm range.

Conclusion: MB is a safe and promising agent used as a photosensitizer in aPDT for skin-infected lesions. There is great variability in the parameters found. Comparisons concerning concentration, irradiation time, and light intensity need to be performed.

KEYWORDS
animal models, antimicrobial photodynamic chemotherapy, methylene blue, photobiomodulation, wounds

1 | INTRODUCTION

Infections are a common complication of the wound-healing process. Moreover, local infections are highly prevalent and can lead to many complications, such as antibiotic overuse and bacterial resistance.[1,2] Recent randomized controlled clinical trials (RCTs) and prospective studies have emphasized the importance of colonization and bacterial infection as factors in the maintenance of skin ulcers and poor healing processes.[3] In addition, Norman et al.,[4] in a recent systematic review, found an important correlation between poor healing in surgical or diabetic foot ulcers and local infections.

Classic therapies for the treatment of infected wounds can be very disappointing and often expensive.[5] Antibiotics have lost their effectiveness owing to abuse, resulting in an increase in the number

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of multidrug-resistant bacteria.[5] Therefore, alternative therapies are urgently needed to treat infected wounds.

The aPDT is a clinical therapy used to treat infectious diseases caused by fungi, viruses, or bacteria. The effect of PDT depends on the activation of a photosensitizing agent (PS) by visible light at a wavelength suitable for its absorption of the photosensitizing agent. PDT promotes the death of target cells through photochemical reactions and ROS formation of reactive oxygen species. Reactive oxygen species damage biomolecules, leading to the death of the target cells.[6,7] Previous animal studies on aPDT with many photosensitizers have reported positive results of this phototherapy for infected wounds. In 2020, Oyama et al.[8] highlights the results of cutaneous animal models of wound PDT treatment. Many second- and third-generation photosensitizers have been developed, such as toluidine-O (TBO), indocyanine green (IGC), and meta-tetra (hydroxyphenyl) chlorin (mTHPC), demonstrating that PDT can ameliorate the healing of infected skin wounds. In that review, most of the studies used the red wavelength range, irradiance ranging from 0.5 to 164 mW/cm^2, and radiant exposure from 1.45 to 450 J/cm^2. By targeting bacterial skin infections in animal models, Sun et al.[9] published a systematic review of 29 studies on aPDT against *Pseudomonas aeruginosa*, *methicillin-resistant S. aureus*, *Escherichia coli*, and *Acinetobacter baumannii*. In addition, many photosensitizers have been identified with promising results in the red-light range, with radiant exposure ranging from 6 to 450 J/cm^2 and irradiance ranging from 84 to 300 mW/cm^2.

Methylene blue (MB) is a blue dye with redox-cycling and cationic thiazine properties and is commonly used in antimicrobial photodynamic therapy (aPDT–MB); it is diluted in an aqueous solution at various concentrations. MB is a phenothiazinium salt that has excellent photochemical properties, with intense light absorption at approximately 630–680 nanometers (nm), and promotes aggregation in the target tissue, depending on the concentration and solution used.[10,11] Since it was discovered in 1876, MB has been used for different and large therapeutic and diagnostic procedures, including human cancer management, urinary tract infections, ifosfamide-induced encephalopathy, sterilization of transfusion blood, Alzheimer's disease, cardiovascular conditions, intraoperative visualization of nerves and mainly for the treatment of pediatric and adult patients with acquired methemoglobinemia, a clinical indication already approved by the U.S. Food and Drug Administration (FDA) using MB in humans.[12-15] Historically, MB was one of the first nonbiological drugs used as an antiseptic agent. In November 2021, 39 clinical trials were registered in the United States (https://clinicaltrials.gov, National Institute of Health – NIH) as active ongoing studies to test the clinical usefulness of MB in different areas of medicine. Moreover, 49 protocol studies have already been completed by 2021.

Human study results have also suggested that aPDT–MB and its derivatives can be used to successfully treat cutaneous infections. One of the most studied applications of aPDT-MB is the treatment of superficial skin lesions.[16] In 2016, Bhatta et al.[17] reported promising results regarding PDT for onychomycosis treatment in an elegant systematic review. This review emphasizes that fluconazole-associated aPDT–MB is a better choice than fluconazole alone for the treatment of simple cutaneous superficial fungal infections. In addition, in studies published by Carrinho et al.[18] and Tardivo et al.,[19] microscopic morphometric evaluation was performed on infected diabetic ulcer areas in humans after aPDT treatment in controlled clinical studies, showing favorable results and demonstrating that aPDT is an effective method for diminishing the need for amputation of infected diabetic feet.

However, although PDT has already been extensively studied in the literature, there are still doubts about its results owing to the variability of the described parameters.[16]

Therefore, based on these premises, the objective of this systematic review was to identify animal studies applying aPDT using MB or its derivatives for the treatment of skin wound infections of different aetiologies and to discuss which PDT protocols can be applied to clinical studies in humans.

2 | MATERIALS AND METHODS

2.1 | Study design

This study is a PRISMA systematic literature review.[20] In this review, the term aPDT–MB was utilized to encompass all published studies concerning noninvasive infectious-targeting protocols. These protocols are based on the administration of MB or its derivatives, followed by photoactivation through irradiation with light corresponding to the absorption spectrum of the photosensitizer (630–680 nm). The protocol was registered on the PROSPERO website before the final data extraction (CRD42021203091). The search was limited to studies published in Portuguese, English, Spanish, or other Latin languages and followed the P.I.C.O. strategy terms described in Appendix A. The search was carried out in August 2021 in the following electronic databases Medical Literature Analysis and Retrieval System Online (MEDLINE) via PubMed, LILACS (Literatura Latino-Americana e do Caribe em Ciências da Saúde/Latin American & Caribbean Health Sciences Literature), Embase (Excerpta Medica Database) via Wiley, and OpenGrey database (available at http://www.opengray.eu/). Two independent authors performed the screening, searches, data extraction, and quality analyses. Any disagreements were resolved by consulting a third commentator. The initial searches in the databases were followed by manual searches of the relevant articles.

2.2 | Eligibility criteria for study selection

This PRISMA review included published animal studies evaluating any aPDT protocol using MB or its derivatives for skin wound infectious conditions in any microorganisms related to human infections. We included controlled and uncontrolled original animal studies, and

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MB data from other previously published systematic reviews about this issue. Studies that analyzed in vitro outcomes prior to clinical outcomes in animal models were included; however, only in vivo outcomes were analyzed. Studies based on split-body randomization were excluded. Conference papers and nonsystematic reviews were also excluded.

2.3 | Data extraction, quality assessment, and data analysis

We report all information about the publication, including journal, year, author names, previous registration of protocol, objectives of the study, sample size, design study, targeted microorganisms, outcomes, side effects, aPDT–MB parameters, and results reported by the authors. Regarding the studies that reported results on several photosensitizers, we extracted only MB or its derivatives data. The quality of all selected preclinical studies was assessed using a checklist of nine items that were modified from the Collaborative Approach to Meta-Analysis of Animal Data from Experimental Stroke (CAMARADES)[21,22] with minimal modifications. Narrative analysis, individual data description, and qualitative synthesis of the extracted data were performed.

3 | RESULTS

Reviewing the findings from the chosen studies in a broad and concise manner, all of them supported the use of aPDT–MB or its derivatives for the selected infections. This positive result was consistent across studies, including those with robust methodology and statistical analysis. However, there was a wide range of parameters observed, including radiant exposure ranging from 12 to 360 J/cm^2, dilutions of MB in saline solution or distilled water, irradiation times spanning from 40 to 3600 s, irradiance typically peaking at 100 mW/cm^2, and wavelengths predominantly falling within the 630–670 nm range. Despite this variability, no adverse effects were reported.

After the initial search, 1842 studies related to the topic were identified. After removing the duplicates, 1041 did not meet the initial inclusion criteria and were excluded. We evaluated 670 titles and abstracts, of which 32 were fully read. Of these, 13 were included in the data extraction, totaling a sample of 261 animals studied[23–35]

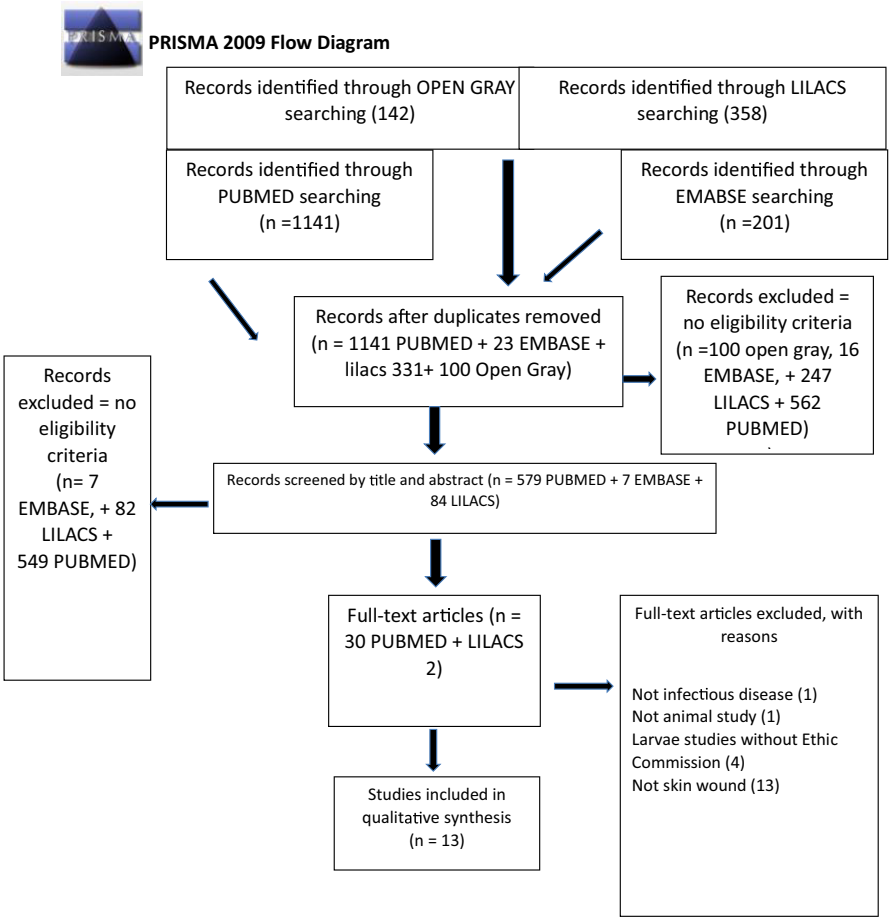

FIGURE 1 PRISMA flowchart of the study selection process.[20]

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(Prism Flowchart, Figure 1). It is important to highlight that the study by Nascimento et al.[24] did not involve animal models. This is a veterinary study. We chose to include this study because it was a veterinary study in which the animals were inserted in a context closer to reality, unlike a laboratory experimental animal model, and the information obtained from this study is very similar to the real occurrence of the injuries, which can provide valuable insights into the applicability of their results. Therefore, we chose not to exclude this study from the data extraction and analysis.

Regarding data extraction, all 13 studies were comparative controlled studies, and six of them described the use of randomization for groups.[23,24,30,31,33–35] No systematic reviews or meta-analyses related to the purpose of this study were found. No study presented a previous protocol registration. No study described the randomization method, allocation, or sample size calculation method, even though five of them mentioned randomization, and 12 studies described details about the statistical analysis of their data.[23–29,31–35] All studies used standardized and objective methods to assess outcomes, and no serious adverse effects were observed in any study. Three studies did not report the number of animals studied. Seven studies evaluated skin infections caused by bacteria,[23,24,26,29–31,35] 4 studies by Leishmania,[25,27,33,34] and 2 studies by fungi.[28,32] The best quality results and the largest number of animals analyzed corresponded to studies on the use of aPDT–MB in superficial bacterial skin infections. Studies of fungal and also bacterial infections have revealed a wide range of genera. We found three studies on *methicillin-resistant Staphylococcus aureus*,[23,26,31] one study on *methicillin-resistant Staphylococcus aureus* (*MRSA*) and *Pseudomonas aeruginosa*,[30] one study on *A. baumannii*,[35] one study on *Candida albicans*,[32] one study on *Sporothrix schenckii* and *S. globosa*,[28] one study on *Pseudomonas aeruginosa*,[29] and one study on multiple gram-positive and gram-negative organisms.[24] The animal species used in the studies were mice (eight studies),[23,25,26,28,30,32,33,35] hamsters (two studies),[27,34] rats (two studies),[29,31] and magellanic penguins (one study).[24] The CAMARADES 9-item quality-adapted checklist was used to assess each study, and the number of criteria met varied from 3/9 to 6/9, with an average of 4.83.

Detailed characteristics of the studies are presented in Table 1. The PDT parameters are listed in Table 2. The quality assessments are presented in Table 3.

4 | DISCUSSION

This study is a PRISMA systematic literature review that was performed to update information on the use of aPDT-MB or its derivatives for treating animal models of skin wound infections, of varied etiologies, with significant human clinical correlations. All studies found results favoring the use of aPDT–MB for the selected infections, demonstrating the superiority of aPDT compared to control groups for one or many reported outcomes. Moreover, among the 11 studies that described a formal statistical analysis of their results, there were favorable results for the use of aPDT-MB in the treatment of wound infections.

Several factors, such as the physical and chemical characteristics of MB, aspects related to optical shielding, photobleaching, light absorption, formulation, concentration, and solvents used, can influence its results.[36] It is also known that more modern photosensitizers, such as New MB (NMB) and 1,9 Dimethyl MB (DMMB), are more phototoxic to different microorganisms than the original composition of MB, probably because they have a more lipophilic nature and a high-cationic charge.[37–39]

Among the studies selected here, we found five that tested newer MB generation PSs or methodologies to modify the characteristics of the MB to improve its anti-infective properties.[26,30–32,35]

Fila et al.[30] compared four PSs, Rose Bengal, porphyrin TMPyP, NMB, and TLD1411, as PSs in a murine model of infected wounds treated with PDT. The NMB exhibited the highest antimicrobial efficacy. Nevertheless, the bacterial irradiance after the end of NMB–PDT returned slower than other PSs, but faster than untreated wounds. Therefore, although there was a clinical reduction in the bacterial load, suggesting that PDT–NMB is a treatment option to be tested in clinical studies, the in vivo antimicrobial effect of NMB was less effective than its in vitro effect.

Ragas et al.[35] tested Toluidine Blue O (TBO), MB, DMMB, and NMB for the photodynamic inactivation of multidrug-resistant *A. baumannii* in their previous in vitro study. All PSs were effective in vitro; however, NMB was the most effective of the four PSs, achieving a 3.2-log reduction in bacterial luminescence during PDT in vivo. Additionally, Dai et al.[32] showed in vitro that NMB was superior to MB and TBO in inactivating *C. albicans* in vitro. Therefore, the author used NMB, instead of the original MB, to conduct the clinical animal study. Despite this choice, the results were dependent on the number of baseline colony-forming units (CFUs) and on the ratio of PS concentration to fungal cell density, which requires higher concentrations of PS. It is known that very high concentrations of PSs can decrease the penetration of light into deep tissue regions, which consequently affects the PDT results. Therefore, even for newer compounds such as NMB, issues related to optical shielding still interfere with the results and are major challenges in the clinical applicability of PDT–MB.

El-Khordagui et al.[31] discovered that electrospun nanofibers (NFs) combined with MB for drug delivery demonstrated enhanced photodynamic activity against wound bacteria. Additionally, Rineh et al.[26] evaluated the NorA efflux pump inhibitor INF55 when added to MB to augment photodynamic therapy (PDT) for infected wounds in vivo. This combination exhibited improved PDT efficacy and enhanced wound-healing effects in a murine model of MRSA-infected wounds.

These findings collectively suggest that altering the properties of MB concerning its interaction with solvents, light, and tissues could enhance therapeutic outcomes. Furthermore, MB has limitations stemming from its physical and chemical properties, indicating that newer photosensitizers may offer superior efficacy compared to MB.

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TABLE 1 Detailed characteristics of the studies.

Author	Year of publication	Study design	Animal	Sample	Analysis	Microorganism	Groups and procedures	Results
Zolfaghari et al.[23]	2009	Randomized Controlled	C57 Black mice	NM	Effect of PDT on the temperature of the wounds; number of viable MRSA isolated per wound, histological sections	*Methicillin-resistant Staphylococcus* aureus	MB alone, light alone group, untreated control group received no MB or light illumination	A25-fold reduction in the number of viable EMRSA was seen This was independent of the increase in temperature of the wounds Histological examination revealed no difference between the PDT-treated wounds and the untreated wounds, all of which showed the same degree of inflammatory infiltration at 24 h
do Nascimento Volpe et al.[25]	2018	Controlled	BALB/c mice	25	Lesion Evolution and determination of Parasite Load	*Leishmania amazonensis*	Gluc+PDT, treated with Glucantime® and PDT; Gluc; PDT; Ampho + PDT; Ampho and control infected but not treated	Statistical differences were not found ($p > .05$) between measures of volume and thickness of the infected footpads in the treated groups when compared with the control group There was a significant reduction ($p < .05$) in the parasitic load of the popliteal lymph nodes of the Gluc + PDT, Gluc, PDT and Ampho groups when compared to the control group In the histological analysis Gluc+PDT group presented a smaller amount of amastigote nests and lower intensity of the mononuclear infiltrate when compared to the Gluc and PDT groups
Nascimento et al.[24]	2015	Randomized Controlled	Magellanic penguins	10	Bacteriological culture and antibiogram plus photographic records of the lesion areas	*E. coli*, *P. mirabilis*, *Pseudomonas* spp., *Pseudomonas. aeruginosa*, *Morganela morganii* *Staphylococcus* spp., *S. aureus*, *Enterococcus* spp	PDT and ointment antibiotics (ATB), systemic antibiotic and anti-inflammatory drugs	There were significant differences in healing rate and average healing time between the PDT and ATB groups (63.62% vs. 9.09% and 42 vs. 70 days, respectively)

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TABLE 1 (Continued)

Author	Year of publication	Study design	Animal	Sample	Analysis	Microorganism	Groups and procedures	Results
Peloi et al.[27]	2010	Controlled	Hamsters	38	Measurement of footpad thickness using a dial thickness gauge (Mitutoyo, Japan) Quantification of parasites in lymph node and spleen	*Leishmania amazonensis*	Control: MB half in lotion and half in water without LED, group A (oil/water (O/W) lotion with 10 nM MB and group B 10 nM MB aqueous solution	The treatment promoted a decrease in the thickness of the infected footpad ($p = .0001$) and reduction in the parasitic load in the regional lymph node ($p = .0007$) of the animals from the group treated with MB + LED PDT using MB + LED
Sbeghen et al.[34]	2015	Controlled	Golden Hasmters	40	The thickness of the footpad (infected and non-infected footpad) with a thickness gauge (Mitutoyo, Japan) and parasite burden in regional lymph nodes and spleen	*Leishmania braziliensis*	Control group: the animals received no treatment. AmB group: the animals were treated intraperitoneally with amphotericin B (5 mg/kg/day) for 20 days with a break of 7 days and further injection for 7 days (positive control). MB-Id group: the animals received 50 μL of 10 mM MB in saline, inoculated intradermally at a single point on the outer edge of the lesion. MB-Tp group: the animals received 50 μL of 10 mM MB in water, applied topically on the lesion. The lesion was illuminated with LED for 1 h	Animals of MB–Tp group presented lesion healing with significant diminution in extent of the lesion, and reduced parasite burden compared to control group; however, no significant difference was seen compared to the AmB group. MB–Tp group also showed reconstitution of the epithelium, the formation of collagen fibers, organization in the epidermis, a little disorganization and inflammation in the dermis. MB-Id was ineffective in all parameters evaluated, and it was comparable to the control group results
Rineh et al.[26]	2017	Controlled	BALB/c mice	NM	Quantitative wound bioluminescence	*Methicillin-resistant Staphylococcus aureus*	Negative control (no compound + light); MB + no light; MB + light; INF55(Ac) en-MB; INF55(Ac) en-MB + light	INF55(Ac)en-MB increased uptake into S. aureus cells and enhanced aPDT–MB activity and wound healing effects

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TABLE 1 (Continued)

Author	Year of publication	Study design	Animal	Sample	Analysis	Microorganism	Groups and procedures	Results
Cabral et al.[33]	2020	Randomized Controlled	BALB/c mice	12	Parasite burden, lesion size, pain, and nociceptive sensivity.	*Leishmania amazonensis*	APDT 1 group, one-session; APDT 2 group, two-session	Although APDT 1 and APDT 2 groups have shown similar parasite burden after 4 weeks, two sessions were clinically better, especially considering the inflammatory process associated
Dai et al.[32]	2011	Controlled	BALBc/mice	11	Fungal luminescence intensity correlated with the corresponding *C. albicans* CFU	*Candida albicans*	Wounds infected with 10×6 and 10×7 CFU were treated 30 min after or 24 h after infection, Control: Nontreated	Control group (no irradiation) doesn't present reduction on fungal luminescence. PDT with NMB in vivo initiated either at 30 min or at 24 h post infection significantly reduced C. albicans burden in the infected mouse skin abrasion wounds
El-Khordagui et al.[31]	2017	Randomized Controlled	Wistar rats	12	Wound healing assessment: morphological, morphometric, microbiological examinations; histopathological examination; PCR analysis of selected wound healing-related genes. Full-thickness inoculated excision wounds in rats	*Staphylococcus aureus ATCC6538P and methicilin resistant Staphylococcus aureus* (*MRSA*)	Untreated control rats kept in the dark (group 1); rats treated with red light (group2); treated with MB solution and red light (group3); treated with MB-eluting PHB/PEG NFs and red light (group 4)	Wound contraction was observed in all cases, the difference in % reduction in wound diameter in groups 2 and 4 compared to group 1 was statistically significant, while the difference between groups 2 and 3 did not reach significance. Wounds in groups 2 and 3 remained heavily infected, significant subsidence in wound infection was observed in group 4 wounds
Fila et al.[30]	2016	Randomized Controlled	Mice Murine model	51	Bacterial counts/ bioluminescence signal saturation	*Staphylococcus aureus methicilin resistant* (MRSA) and *Pseudomonas arginosa*	6 groups: noninfected mice; cover with Tegaderm dressing; no dressing; removal of dressing at the end of day 2; bacterial strains for each of the four PSs; evaluated PDT efficacy versus potential LLLT effect	All PS tested in vivo (including NMB) showed decrease in UFC amount. NMB had the highest BLI radiance reduction, however, regain was faster than in untreated wound. In vivo the growth delay was limited with 24–48 h in pathogen expansion for MRSA, and noticed longer growth suppression of P. aeruginosa with TLD1411 mediated PDT

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TABLE 1 (Continued)

Author	Year of publication	Study design	Animal	Sample	Analysis	Microorganism	Groups and procedures	Results
Li et al.[28]	2019	Controlled	BALBc/ mice	NM	Decrease of CFU, morphological and molecular analysis by Image J, molecular genetic analysis and culture test	*Sphorothrix schenckii*, *S. globosa*	Itraconazole; PDT; itraconazole + PDT; sodium chloride 0.9%	The size of the lesions on day 30 was smaller than day 20, in all groups. The group itraconazole + PDT showed most remarkable reduction, in which the skin of some mice had become normal, before day 20. Group itraconazole+PDT presented negative results to fungal culture on day 30; the other groups – control, itraconazole and PDT – the results to fungal culture were positive
Krasnoselskiy et al.[29]	2019	Controlled	WAG rats	50	Quality of healing of an infected radionuclide was determined by comparing the histological and morphometric study of skin, measure the size of the lesion, and CFU	*Pseudomonas aeruginosa*	Skin ulcer+infection+PDT; skin ulcer; skin ulcer + infection	Control group I – the vertical section area reduced up to 73% by the day 30, and 51% by the day 52. Control group II – the area of vertical section in the infected radiation ulcer reduced 30%; the vertical section area on noninfected ulcers was 50% reduced at day 52. Group III – at day 52 the vertical section area cavity, in infected ulcers, was absent due the complete healing of the radiation ulcer
Ragas et al.[35]	2010	Controlled	BALB/c mice	12	Differences in the bioluminescence–time curve	*A. baumannii*	PDT (MB, NMB, DNMB, TBO) treated mice and untreated burns	NMB was the most effective of the four dyes, achieving a 3.2-log reduction of the bacterial luminescence during PDT in vivo A statistically significant reduction of the area under the bioluminescence–-time curve of PDT-treated mice was observed

Abbreviation: NM, not mentioned.

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TABLE 2 PDT parameters of stimulation.

Author	Dilution	System light	Time of irradiation	Power/Irradiance	Diameter	Radiant exposure/energy	Model of application	Frequency, sessions number
Zolfaghari et al.[23]	100 μg/mL	Diode laser (PerioWave system, Ondine Biopharma, Vancouver, Canada)	1800 s	200 mW distributed by a fiber optic cable and a diffusing head	1 cm^2 circle of illumination	360 J/cm^2	The source was held at a constant distance from the wound to produce a 1 cm^2 circle of illumination	One session
Do Nascimento Volpe et al.[25]	0.50%	Red LED system	1800 s (first cicle) and 3600 (second cycle)	2.63–4.27 mW/cm^2	Not reported	Not reported	Two lighting systems were having a plate of red LED formed by 6 rows of light emitting diode (LED) units with 75 cm total length and 3 cm width (2.63 10μ^3Watt/cm^2), grasped by a wooden base at the sides of the board, distant 4 cm (in the first system) and 1 cm (in the second system −4.27 10μ^3Watt/cm^2) of the LED. Between the LEDs and the animals' footpad a metal screen was put to support the animal	Performed in 2 cycles: first cycle the first lighting system was used and, 45 days after the lesion development the 6 groups were treated with lesions lasted 30 min with sequent irradiation with red LED for 30 min, performed twice a week for 4 weeks. After 105 days of infection, Gluc+PDT, PDT and Ampho+PDT groups were submitted to the second cycle of treatment, when the second lighting system was used with more time in contact between MB and lesion (1 h) and more irradiation time (1 h) irradiating 3 times a week for 4 weeks
Nascimento et al.[24]	300 μM	Laser RECOVER (MM Optics)	40 s per point	Power, 100 mW; irradiance/point, 3.3 W/cm^2	Not reported	133.3 J/cm^2, 4 J/point	Applied at 1 cm equidistant points perpendicular and in contact to the lesion, as many as were needed to cover the wound	Three times a week
Peloi et al.[27]	10 nM	The LED (EverLight Co.) light system was constructed using 6 units (in series) that emit red light, and their individual output was determined using a Handheld Laser Power Meter (Edmund Optics Inc.)	3600 s	5 mW/cm^2 each LED was used for 1 h	Not reported	12 J/cm^2	Not reported	Three times a week
Sbeghen et al.[34]	10 mM	LED (Everlight) system containing 6 units (in a serie) that emit red light, with 663 nm wavelength	3600 s	5 mW/cm^2 each LED was used for 1 h	Not reported	18 J/cm^2 each LED	Not reported	Three times a week, 36 session
Rineh et al.[26]	MB and INF55(Ac) en – MB 12 (40 μL of 200 μM stock solutions)	Red light	1200 s total (each radiant exposure with a different time)	300 mW, with spots positioned at the required distance from animals to give an irradiance of 100 mW over the 1.0 cm^2 wound area	1.0 cm^2	12, 36, 84, 108, and 120 J/cm^2 depending on time irradiation	Red light was applied in 5 doses over 20 min	Mice were then illuminated with 652 nm light in 2, 4, 8, 4, and 2 min aliquots over a 20 min period, corresponding to each different fluence

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TABLE 2 (Continued)

Author	Dilution	System light	Time of irradiation	Power/Irradiance	Diameter	Radiant exposure/energy	Model of application	Frequency, sessions number
Cabral et al.[33]	100 μM	Red LED	1500 s	100 mW/cm^2	Not reported	150 J/cm^2	Not reported	One-session 1×/day or two-session one and 24 h after the first day
Dai et al.[32]	400 μM NewMB (NMB) (556416-1G; Sigma)	Red light Light was delivered topically using a noncoherent light source (Luma Care, Newport Beach, CA)	Variable	32.5 mW/cm^2	Not reported	78 J/cm^2 (for PDT at 30 min post-infection) or 120 J/cm^2 (for PDT at 24 h post-infection)	Contact	One-session initiated either at 30 min or at 24 h after fungal inoculation
El-Khordagui et al.[31]	Polyhydrohybutyrate/polyethylene glycol (60:40 PHB/PEG) polymer blend and methylene blue (MB – NFs) or MB alone	Red light, 635 nm	25 min for 100 J/cm^2 and 50 min for 200 J/cm^2	150 mW	The lamp-wound distance was adjusted to approximately 3 cm to ensure uniform exposure of the whole wound and its edges (an area of approximately 16 mm diameter) to the same light intensity	Groups 2–4 were irradiated with a total light dose of 700 J/cm^2 according to: 100 J/cm^2 on days 0, 1, and 3 and a booster dose of 200 J/cm^2 on days 7 and 8. Rats in group 4 were treated with an NFs mat providing 60 μg of MB on days zero, 7 and 8. A similar MB dosing and irradiation schedule was adopted in the PDT–MB group 3	The lamp-wound distance was adjusted to approximately 3 cm	Different according to the stimulated group
Fila et al.[30]	500 μM	A custom-built LED light source emitting (manufactured by Theralase Inc. Toronto, ON, Canada) 525 nm	2220 s	50 mW/cm^2	Not reported	100 J/cm^2	Not reported	One session of 37 min
Li et al.[28]	Not reported	A light-emitting diode (LED) lamp Yage Optic and Electronic Technique, Wuhan, China)	1800 s	80 mW	3.6 cm^2	40 J/cm^2	Not reported	One time/week, during tree weeks, a distance of 10 cm from the source for 30 min
Krasnoselskiy et al.[29]	0.1% solution	Photon apparatus "Barva & LED/630"	Not reported	Not reported	Not reported	45 J/cm^2	Not reported	One session
Ragas et al.[35]	1 mM	Red light was delivered using a noncoherent light source with interchangeable fiber bundles (LumaCare, Newport Beach, CA)	Not reported	100 mW/cm^2	Not reported	Up to 360 J/cm^2 in aliquotes (results obtained for MB at 180 J/cm^2)	Not reported	One session

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TABLE 3 The adapted CAMARADES Quality Assessment checklist.

Author	Publication in peer-reviewed journal	Statement of control of temperature	Compliance with animal welfare regulations	Randomization of treatment or control	Allocation concealment	Blinded induction model	Blinded assessment of outcome	Sample size calculation	Statement regarding possible conflict of interest	Total (Y on 09 itens)
Zolfaghari et al.[23]	Y	Y	Y	Y	Y	N	N	N	Y	6
Do Nascimento Volpe et al.[25]	Y	Y	Y	N	N	N	N	N	Y	4
Nascimento et al.[24]	Y	Y	Y	Y	Y	N	N	N	N	5
Peloi et al.[27]	Y	Y	Y	N	N	N	N	N	N	3
Ragas et al.[35]	Y	Y	Y	N	N	N	N	N	N	3
Sbeghen et al.[34]	Y	Y	Y	N	N	N	N	N	Y	4
Rineh et al.[26]	Y	Y	Y	N	N	N	N	N	Y	4
Cabral et al.[33]	Y	Y	Y	Y	Y	N	N	N	N	5
Dai et al.[32]	Y	Y	Y	N	N	N	N	N	Y	4
El-Khordagui et al.[31]	Y	Y	Y	Y	Y	N	N	N	N	5
Fila et al.[30]	Y	Y	Y	Y	Y	N	N	N	Y	6
Li et al.[28]	Y	Y	Y	N	N	Y	Y	N	Y	6
Krasnoselskiy et al.[29]	Y	Y	Y	N	N	N	N	N	N	3
Total (Y on 13)	13	13	13	5	5	1	1	0	7	

However, even if MB is less active than other phenothiazinium dyes, such as DMMB, NMB, or TBO,[40] even if studies show that the effectiveness of MB as an anti-infective agent in vivo is inferior to its in vitro results, probably due to the physicochemical characteristics of this photosensitizer, even if it is already known that MB has its effectiveness reduced relative to the efflux systems of bacteria,[41] MB has some very favorable characteristics for its use, such as not causing carcinogenic or mutagenic effects, high selectivity for tumors or microorganisms, stability and economic viability, greater absorption in soft tissues in visible light, and well-defined structure and chemical purity.[10,11] Therefore, MB is widely used in clinical practice.

In the clinical results of the animal studies selected here, concerning PDT–MB parameters, there was great variability among the included studies, and these data are summarized in Table 2. As we did not find more than one study with the same population, methodology, treatment, and outcome, we restricted our analysis of the results to qualitative rather than quantitative discussion. Therefore, it was not possible to provide a meta-analysis of the results to determine the best parameters related to aPDT–MB in infectious skin diseases.

In this review, the best quality results and the largest number of animals analyzed corresponded to studies on the use of aPDT–MB in superficial bacterial skin infections.[23,24,26,29–31,35] We also found interesting results in four animal studies that evaluated the use of PDT for the treatment of animal cutaneous leishmaniasis.[25,27,33,34] These studies showed a decrease in both the parasite load and the size of the lesions, suggesting that PDT can be used as an adjuvant in the treatment of tegumentary leishmaniasis, thereby reducing the total time of formal treatment, which is a promising result.

Regarding studies that reported data on bacterial and fungal wound infections, irradiance ranged from 50 mW/cm^2 to 3.3 W/cm^2, most commonly with a maximum of 100 mW/cm^2, radiant exposure ranging from 12 to 360 J/cm^2 and MB diluted in saline solution or distilled water. In studies on leishmaniasis infections, the radiant exposure ranged from 12 to 150 J/cm^2, and irradiance ranged from 2.63 to 100 mW/cm^2.

It has been suggested that the best parameters for human use of aPDT–MB for bacterial and fungal infections are irradiances ranging from 50 to 750 mW/cm^2 and radiant exposures from 6 to 18 J/cm^2, with other parameters being undefined. Recently, Shen et al.[42] evaluated the safety and efficacy of aPDT–MB in infected wounds of five patients, all of whom were treated with aPDT–MB using red LED irradiation (635 nm, 120 J/cm^2, and 100 mW/cm^2). After an average of four PDT sessions, the infected wounds in all the patients were resolved. As shown by our results, previously published human clinical studies were based on red visible light, lasers, or LEDs.

Regardless of this parallelism, the variability in the values found in animal and human studies remains very large. In addition, previous in vitro studies have highlighted that the best cytotoxic effects of aPDT–MB are related to the concentration of the photosensitizer, irradiation time, and light energy. Low-dose photodynamic therapy has been studied with regard to the healing of wounds,[43] infections,[44] and skin lacerations[45] with excellent results despite its low-dose therapeutic parameters. However, the efficacy of

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low-dose photodynamic therapy remains controversial. In a recent study, Zuhayri et al.,[46] using an optical coherence tomography (OCT) in vivo evaluation, showed that even using low irradiation energies (1–4 J/cm^2) by an AlGaInP laser (λ = 630 nm, P = 5 mW) with saline MB 0.01%, the best results to treat infected wounds in mice were obtained with the highest intensity of irradiation.

In the extracted studies, studies were carried out at a lower frequency of sessions and follow-up time, often being only single, two, or three sessions per animal, with a lower dosage of stimulation parameters, and all of which showed positive results, suggesting that an even more restricted range of stimulation parameters can produce better results. Regarding light parameters, three studies on leishmaniasis used 2.63–5 mW/cm^2, and only one study used more than 100 mW/cm^2 of irradiance. Additionally, regarding bacterial infections, only one study performed PDT more than once a week, with a follow-up time of more than 1 week, and had positive results.

Therefore, issues related to aPDT–MB dosimetry are multifactorial and complex and depend not only on stimulation parameters but also on the purpose of therapy, the environment, and the time of injury. This general rule holds true for both the therapeutic effects and toxicity. It is also important to point out that not all studies have evaluated changes in the healing process, inflammation, or lesion size beyond bacteriological quantification. Furthermore, two studies showed improvement in bacteriological parameters without healing improvements. This aspect negatively affects these results because the treatment of infection in these lesions aims, in addition to preventing the spread of infection, to accelerate the healing process. Four distinct stages of wound healing have been described, from the hemostatic to the maturation phase. Efficient healing of ulcers is intrinsically related to the local treatment of possible cutaneous infections. Then, many factors can affect wound healing, including intrinsic local infections, which delay the healing process.[47,48] Thus, it is essential that these studies evaluate the healing process of skin ulcers concomitantly with improvements in the infection process. Ideally, aPDT for bacterial lesions should yield positive results for both outcomes.[9] Only six studies[24,27–29,33,34] found positive changes in both parameters, with radiant exposure ranging from 12 to 133.3 J/cm^2 in four studies and 150 J/cm^2 in one study. In this way, a long-term follow-up period needs to be added in future studies to better evaluate this important but neglected outcome, to underline the percentage of improvement in relation to the initial size and depth, and to understand the side effects of using more energy irradiation.

Finally, the photodynamic effect depends on oxygen, and in general, patients with skin lesions associated with peripheral arterial disease (PAD) may present results of a lesser magnitude than those found in animal models of nonvascular infected ulcers. Therefore, further studies, including animal models of vascular diseases associated with infections, are required.[49,50]

Thus, although all studies were controlled, the control groups included other therapies, such as known standard treatments or other pharmacological therapies. Therefore, although the results of this study converge and reinforce the promising results of aPDT-MB, each study used different therapeutic parameters. Thus, future studies comparing different aPDT parameters, such as concentration, irradiation time, and light intensity, are still required.

5 | CONCLUSION

This is the first systematic review focused on aPDT based on an MB photosensitizer for the treatment of infected skin wounds in animal studies. This systematic review suggests that RCTs can be conducted to evaluate of aPDT–MB in superficial fungal infections, bacterial skin infections, and tegumentary leishmaniasis by comparing aPDT–MB with known standard treatments. Our results showed great variability in the reviewed animal studies concerning PDT parameters, suggesting radiant exposure from 12 to 360 J/cm^2, MB diluted in saline solution or distilled water, irradiation time from 40 to 3600 s and irradiance most commonly at a maximum of 100 mW/cm^2, depending on the etiology of the lesion, its size, and depth. These results also reinforce that MB is a safe and promising agent for use as a photosensitizer in antimicrobial PDT for skin-infected lesions. Therefore, it is urgent to conduct comparisons of the concentrations used, irradiation times, and light intensities in the same study.

AUTHOR CONTRIBUTIONS

Ana Paula Martin Cardozo: Conceptualization, Writing – original draft, Methodology, Project administration, Validation, Formal analysis, Writing – review & editing. Daniela de Fatima Teixeira da Silva: Conceptualization, Methodology, Writing – review & editing, Supervision, Resources. Rita de Cassia Ferreira: Visualization, Writing – review & editing. Kristianne Porta Santos Fernandes: Methodology, Validation, Writing – review & editing. Adriana Lino-dos-Santos-Franco: Resources, Writing – draft and review & editing. Maria Fernanda Setúbal Destro Rodrigues: Resources, Writing – draft and review & editing. Lara J Motta: Methodology, Data curation, Formal analysis, Resources, Writing – review & editing. Rebeca Boltes Cecatto: Conceptualization, Project Administration, Methodology, Supervision, Data curation, Supervision, Resources, Writing – original draft, review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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APPENDIX A

PUBMED SEARCH STRATEGY

("1995"[Date – Entrez] AND ("Methylene Blue/administration and dosage"[Mesh] OR "Methylene Blue/organization and administration"[Mesh]OR"MethyleneBlue/pharmacokinetics"[Mesh] OR "Methylene Blue/pharmacology"[Mesh] OR "Methylene Blue/physiology"[Mesh] OR "Methylene Blue/radiation effects"[Mesh] OR "Methylene Blue/standards"[Mesh] OR "Methylene Blue/therapeutic use"[Mesh] OR "Methylene Blue/therapy"[Mesh]) OR (methylene blue AND standard protocol) OR (methylene blue AND exposure time AND photodynamic therapy) OR (methylene blue AND exposure time) OR (methylene blue AND energy and photodynamic therapy) OR (methylene blue AND radiant exposure) OR (methylene blue AND irradiance) OR (methylene blue AND power density) OR (methylene blue AND power AND photodynamic therapy) OR (methylene blue AND operating mode) OR (pre-irradiation time AND methylene blue) OR ethylene blue OR methylthioninium chloride OR swiss blue OR blue n OR methylene blue OR methylthionine chloride OR prolene blue) AND (photodynamic therapy OR photo* OR photobiomodulation OR photochemotherapy OR photosensitizer OR photosensitization) AND (antimicrobial OR antibacterial OR infection OR infectious OR infected OR antiseptic OR disinfection OR microbial OR infec*)

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