

Methylene blue and its importance in medicine

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ABSTRACT

Methylene blue (MB), a blue-colored aniline-based dye that was first developed for the dyeing of cotton, is a phenothiazine derivative that is soluble in water and organic solvents. Its reduced form, leucomethylene blue, is colorless and has a regulatory effect on the redox cycle. This feature allows it to be used in malarial therapy by inducing glutathione reductase (GR), an enzyme of glutathione metabolism. Malaria is a disease characterized by repeated growth cycles of Plasmodium group parasites in erythrocytes and changes in glutathione metabolism. Methylene blue was recognized as an antimalarial agent when it was observed that it reduced reactive oxygen species (ROS) by inhibiting Plasmodium falciparum glutathione reductase and by selectively inducing oxidative stress. It has also been observed that MB can reduce factors that cause aging and Alzheimer's disease, such as the accumulation of tau proteins in plaques, mitochondrial dysfunction, and ROS increase due to disorders in the electron transport chain (ETC). Methylene blue has been used in Alzheimer's treatment, considering its features of tau protein inhibition, anti-ROS antioxidant properties, and ability to regulate ETC. Methylene blue, known for its antimalarial and antioxidant effects, has also been used in the treatment of Candida infections as an antifungal agent. In candidiasis infections, MB is aimed to cause mitochondrial dysfunction in yeast and treat the patient. In this review, the history of MB as an antimalarial, antioxidant and antifungal agent; malaria, Alzheimer's, and its role in the treatment of candidiasis. In this review, the history of MB and its role as an antimalarial, antioxidant, and antifungal agent in the treatment of malaria, Alzheimer's, and candidiasis are discussed.

Keywords: Alzheimer's disease, antifungal, antimalarial therapy, candidiasis, electron transport chain, glutathione reductase, methylene blue, mitochondrial dysfunction, tau proteins.

Methylene blue (MB, methylthioninium chloride), a phenothiazine known for its ability to cross the blood-brain barrier and exert neuroprotective effects, is considered a potential therapy, especially for neurodegenerative diseases.^[1,2] Among its beneficial properties, MB is a redox cycle stabilizer and an electron donor.^[1] Studies have found that MB can be both a substrate and an inhibitor of glutathione reductase, which is an important enzyme of glutathione metabolism,^[3] and its ability to target Plasmodium falciparum glutathione reductase (PfGR) instead of human glutathione reductase (hGR) at therapeutic concentrations has attracted considerable attention in antimalarial therapy.^[4] In addition, MB is also used in the treatment of

Alzheimer's disease (AD),^[5] which occurs as a result of mitochondrial dysfunction, neuroinflammation, and oxidative stress. It is also known that defects in mitochondrial functions, which are responsible for energy production in the cell, occur before the accumulation of amyloid β (A β) that causes AD and are closely related to the accumulation of tau protein.^[6,7] Studies have shown that MB can delay aging by inhibiting the accumulation of tau protein^[8] and by increasing mitochondrial respiration,^[9] hence it is considered a significant chemical in regards to AD. Candidiasis, an infection caused by Candida spp.,^[10] is the result of carbohydrate-active enzymes acting on the cell wall which lead to wall remodeling, changes in stress resistance, and extracellular matrix

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accumulation.^[11] They usually cause infection by forming biofilms on implanted medical devices.^[12] Many studies have attempted to prevent multiple drug resistance that develops after interventions used to eliminate candidiasis (surgery and antifungal agents).^[13] While MB is known for causing mitochondrial dysfunction and changes in redox cycling,^[5] its use in candidiasis treatment as well as its antifungal and antioxidant properties have also been recognized.^[14] In addition to these diseases, MB is also involved in the treatment of methemoglobinemia, encephalopathies, and neurodegenerative disorders.^[15] This review discusses the role of MB only in the treatment of malaria, AD, and candidiasis.

METHYLENE BLUE

Methylene blue was first synthesized in 1876 by the German chemist Heinrich Caro (1834-1911) as an aniline-based dye for cotton dyeing.^[16] Methylene blue (chemical name: tetramethylthionine chloride) is a cationic dye and belongs to the class of compounds known as phenothiazines (neuroleptic drug group).^[17] It is soluble in water and organic solvents.^[18] It is in the oxidized state and dark blue in color, showing maximum absorption at 609-668 nm wavelengths. Since it cannot be absorbed in the visible region, it is reduced to leucomethylene blue (leucoMB) and becomes colorless.^[19] These two forms of dye exist in equilibrium as a redox pair; together they form a reversible oxidation-reduction system or electron donor-acceptor pair.^[9]

In 1885, Paul Ehrlich published an article on the staining of the tuberculosis bacillus, called the "side-chain theory", which described the theory of affinity of tissues for various chemical compounds.^[23] According to the side-chain theory, the interaction between synthetics is chemical by nature of compounds and tissues and involves a chemical reaction. In other words, the biological effect of any substance depends on the chemical structure of the substance and the cell. Ehrlich studied the staining intensity of various organs by injecting MB into live animal tissue.^[23] He conducted similar experiments in different animal species, using other synthetic dyes. As a result of these experiments, he proposed that MB has a selective affinity for nerve tissue due to its stabilizing properties on redox

cycling.^[8] Considering the side-chain theory, Ehrlich suggested that if pathogens could be stained with MB, such staining could have a certain detrimental effect on the pathogen.^[24] Methylene blue began to be used as a drug to treat various diseases to eliminate only pathogens, without harming other tissues.^[25] Furthermore, MB also plays an important role in many diseases such as neurodegenerative and psychiatric disorders (Figure 1) and *Candida albicans* infections.^[26]

METHYLENE BLUE IN ANTIMALARIAL TREATMENT

Malaria is a disease characterized by repeated growth cycles of Plasmodium group parasites (*P. falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi*, *P. ovale*) in erythrocytes. Various cellular and molecular strategies allow the parasite to escape

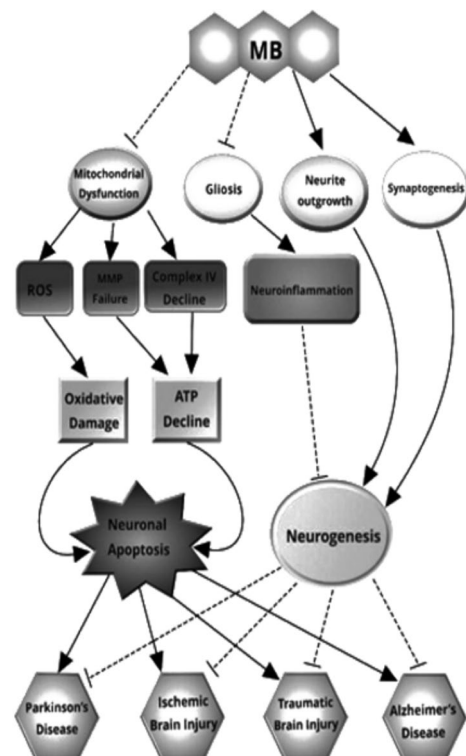


Figure 1. The role of MB in neurodegeneration. Methylene blue can protect against neuronal apoptosis by preventing mitochondrial dysfunction and subsequent oxidative damage and ATP loss. Methylene blue supports neurogenesis by improving neuroinflammation and promoting neurite outgrowth and synaptogenesis. In this manner, MB can prevent neuronal damage and facilitate neuronal repair.^[26]

from the human immune response so they can go through multiple reproductive cycles.^[27] During the erythrocyte cycle, soluble products of *Plasmodium spp*, known as malarial toxins, guide the systemic release of proinflammatory cytokines (molecules that control immunological events such as activation, proliferation of immunocompetent cells, and regulation of cells in tissues) that act on many other cellular systems such as the endothelium.^[28]

After *Plasmodium falciparum* glutathione reductase (PfGR) emerged as a new drug target,^[29] interest in MB as an antimalarial drug increased when MB was also found to be an inhibitor and substrate of glutathione reductase.^[3] Glutathione is an antioxidant that can prevent damage to cellular components by reactive oxygen species (ROS) such as free radicals, peroxides, lipid peroxides, and heavy metals;^[30] it exists in reduced (GSH) and oxidized (GSSG) states. The ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) in cells is a measure of cellular oxidative stress.^[31,32] Glutathione reductase (GR) enzyme plays a role in regulating intracellular redox balance (Figure 2),^[20-22] preserving macromolecules such as proteins, and controlling glutathione-dependent antioxidant systems;^[33] intracellular SH/SS ratio^[34] is maintained by increasing GSH/GSSG ratio.^[22] Like redox cyclers, MB uses glutathione reductase to regenerate^[35-37] and was found to target PfGR rather than human glutathione reductase (hGR) at therapeutic concentrations.^[4] Studies have shown that the development of resistance to MB is difficult *in vivo* activity,^[38] and that *P. falciparum* and *P. vivax* have strong *in vivo* activity against drug-resistant isolates.^[39,40] It was also found that MB has a substantial effect on gametocyte reduction in *P. falciparum*.^[41] For this reason, methylene blue was found to be a potentially useful partner drug for artemisinin-based combination therapy (ACT; a treatment method in which potent drugs are used in combination with artemisinin), especially when elimination is the ultimate goal.^[42]

MITOCHONDRIAL DYSFUNCTION AND METHYLENE BLUE IN ALZHEIMER'S TREATMENT

The brain is dependent on oxidative metabolism as an energy source, consuming 20% of the body's glucose and 20% of oxygen

at resting state.^[43] A large portion of this energy requirement consists of maintaining resting membrane potential, forming action potentials, and postsynaptic effects of glutamate.^[44,45] Considering the damage to the brain as a result of deficiency of these energy sources, proper mitochondrial function is considered crucial to the brain. In contrast, dysfunctional mitochondria cause neurodegenerative conditions.^[46] Mitochondria are major organelles in neurons.^[47] and generate energy as adenosine triphosphate (ATP) via oxidative phosphorylation or the mitochondrial respiratory chain.^[48,49] Other functions of mitochondria include regulation of calcium homeostasis, formation of free radicals, and apoptosis.^[50] Mitochondrial dysfunction results in problems such as changes in mitochondrial respiratory chain enzymes, formation of reactive oxygen species (ROS), opening of the mitochondrial permeability transition pore (mPTP), structural abnormalities of the mitochondria, oxidative stress, and

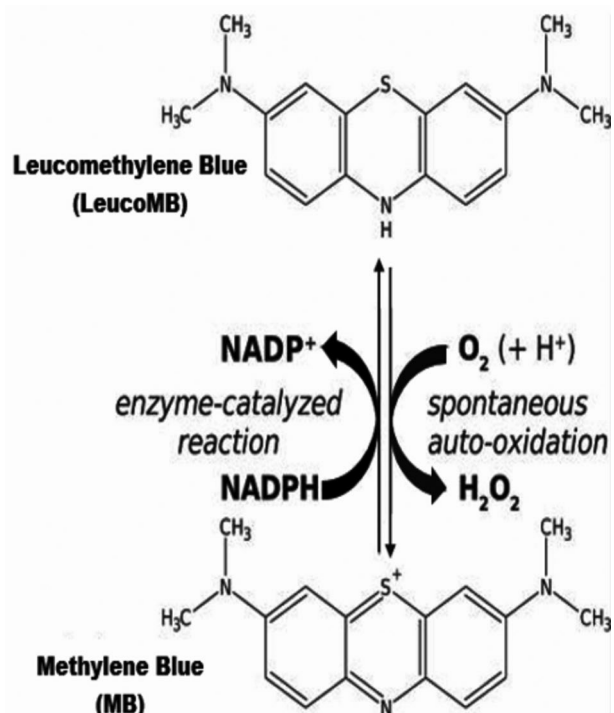


Figure 2. Methylene blue uses glutathione reductase (an enzyme of glutathione metabolism that maintains the -SH group in proteins by reducing oxidized glutathione [GSH] in the cell^[20,21]) as a substrate and transforms to leucomethylene blue; leucomethylene blue is an auto-oxidizer and spends O₂ in each cycle to reduce O₂.^[22]

apoptosis.^[51-53] Proper mitochondrial function depends on the successive passage of electrons at each step of the electron transport chain (ETC).^[54] The primary function of mitochondrial ETC is the step by step transfer of high-energy electrons from food-derived energy substrates such as NADH to O₂. At each step, the energy used to carry protons is released and ATP-synthase synthesizes ATP from ADP by creating a proton gradient between membranes.^[54,55] When the ETC is fully occupied, electron carriers begin to transport electrons to O₂ producing harmful reactive oxygen species (ROS).^[54] The greatest risk for neurodegenerative diseases such as AD is aging, and the accumulation of mitochondrial DNA (mtDNA) mutations and ROS production, which are also observed in Alzheimer's pathogenesis, have been observed to cause aging.^[52]

Alzheimer's disease is a progressive neurodegenerative disease characterized by decreased cognitive functions, self-care deficiencies, and various neuropsychiatric and behavioral disorders due to neuron and synapse losses in various parts of the central nervous system (CNS).^[56,57] Approximately 5-10% of cases are genetic and emerge in an early onset, autosomal dominant manner. Three proteins are known to be associated with such genetic events: amyloid precursor protein (APP), and presenilin 1 and 2 (PS 1 and PS 2).^[52] Alzheimer's disease involves extracellular accumulation of A β derived from APP, in senile plaques composed of hyperphosphorylated tau protein.^[58,59] Amyloid precursor protein is metabolized to A β in two ways. The first of these, the non-amyloidogenic pathway, is the metabolic pathway involving β -secretase and β -secretase enzymes that break down APP, and the products formed are not toxic. In the second pathway, the amyloidogenic pathway involves the formation of A β 1-40 and A β 1-42 derived from β -secretase and β -secretase enzymes.^[60,61] A β 1-40 constitutes approximately 90% of A β . However, the main component of amyloid accumulation is A β 1-42.^[62] Therefore, neurotoxic A β derivatives that form in the amyloidogenic pathway accumulate in amyloid plaques, causing damage to synapses and hyperphosphorylation in tau proteins. It has been observed that mutations in the PS 1 gene cause toxic A β production and hyperphosphorylation

of tau proteins as a result of improper cleavage of APP, triggering the formation of neural fibrillar tangles (NFT).^[63]

The therapeutic research of AD first targeted A β , and MB has been studied since 2007.^[64] In a study on transgenic mice (3 \times Tg-AD), it was observed that MB mediated the proteolytic clearance of A β by increasing chymotrypsin and trypsin-like proteasome (large protein structure) activity in the brain.^[65] In another transgenic mouse (APP/PS1) model, reduced A β accumulation in the hippocampus and adjacent cortex was observed, providing protection against cognitive decline in behavioral tasks that measure social interaction, learning and memory, as well as exploratory activity.^[66] These results obtained from APP/PS1 mice has been attributed to the anti-amyloidogenic mechanism and reduced β -secretase activity and expression.^[67]

Mitochondrial dysfunction in Alzheimer's is determined by a decrease in complex IV, a decrease in other TCA cycle (citric acid cycle) enzymes, and mutations in mtDNA. Degradation in mitochondrial complex IV increases the production of free radicals and oxidants such as hydrogen peroxide (H₂O₂). Lack of energy is a severe consequence of impaired mitochondrial function. Aging also plays a role in neural dysfunction.^[68] It is suggested that research to find therapeutic strategies to increase mitochondrial function may delay aging.^[69]

Methylene blue is the first chemical that can cross the blood-brain barrier^[70] and induce the mitochondrial respiratory complex.^[68] Methylene blue, applied in low doses, exhibits strong antioxidant properties, and its oxidized and reduced forms are balanced in low concentrations (Figure 2). Thus, it protects against oxidative damage and ensures the continuation of mitochondrial function.^[71] Methylene blue prevents aging and neurodegeneration by affecting mitochondria and cytochrome c, through its cycling between its oxidized and reduced forms.^[24] By penetrating the cellular and mitochondrial membranes, MB aggregates in the mitochondria and increases mitochondrial respiration by transferring electrons to oxygen in the ETC. Cytochrome c oxidase is the last member of the electron transport chain, and decreased activity of this enzyme has been reported in AD. Methylene blue induces

cytochrome c oxidase and increases its enzymatic activity, therefore increasing oxidative metabolic capacity of neurons and allowing ATP production in the brain by consuming more oxygen. Methylene blue also increases the production of cytochrome c oxidase.^[9] The potential use of MB in the treatment of AD has also been suggested because of its features of tau filament formation and its inhibitory properties on A β aggregation (Figure 3).^[72] Amyloid β aggregation occurs by a mechanism consisting of multiple pathways, comprising of A β aggregate derivatives such as oligomers, protofibrils, and circular protofibrils. It has been observed that the addition of MB to previously formed oligomers causes oligomer loss and oligomer formation is inhibited due to increased fibril formation.^[64]

These mechanisms of effect indicate that MB displays its therapeutic effect on neurodegenerative diseases through various pathways.

CANDIDA ALBICANS AND THE USE OF METHYLENE BLUE

Candidiasis is a common fungal infection that occurs in the skin, oral cavity and esophagus, gastrointestinal system, vaginal, and vascular system in humans caused by the *Candida* genus of yeast.^[73] *Candida* species are the cause of the fourth most common hospital-acquired bloodstream infections received in the USA and systemic candidiasis has a high mortality rate of up to 50%.^[74] Although only

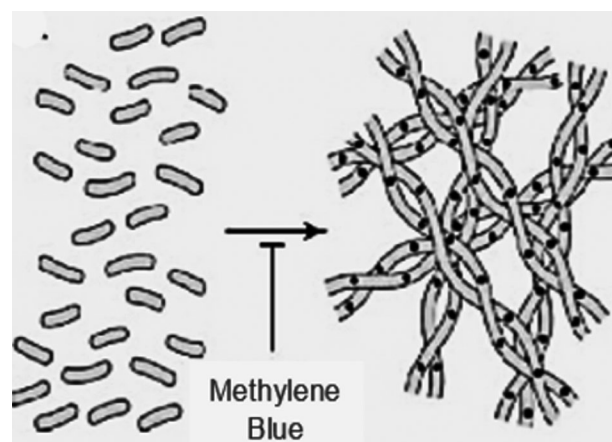


Figure 3. Inhibition of tau aggregation. Methylene blue prevents the formation of tau aggregates called paired helical filaments.^[8]

five species cause the vast majority of *Candida* infections (*Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei*),^[75-79] *Candida albicans* is most responsible for infections.^[73] Differences in *Candida* spp. colonization and survival strategies have been associated with certain risk groups, indicating that they only promote infections under different preconditions. For instance, infections associated with *C. glabrata* and *C. tropicalis* are more common in patients with hematological or solid organ malignancies and neutropenia. *Candida krusei* infections occur especially in patients who have undergone hematopoietic stem cell transplantation. *Candida parapsilosis* is associated with infections in newborns rather than adults and is a common pathogen of catheter-related infections.^[80] *Candida albicans* is a commensal organism that is usually found in the human body but causes superficial and systemic infections in immunocompromised conditions.^[81] It also draws attention as a highly versatile organism, with the ability to survive in a wide variety of settings, each with their own unique environmental conditions. This ability grants *Candida albicans* and *Candida* spp. an advantage over other microorganisms. With limited nutrients on mucosal surfaces, competition between bacteria and fungi (microbiota) provide selective conditions causing less adapted microorganisms to be eliminated. Most bacterial microbiota are known to regulate their catabolic and anabolic pathways in states of limited or excessive nutrition, but have not been characterized despite the knowledge of parallel systems existing among human pathogenic fungi.^[82] For instance, most *Candida*-specific genes (compared to *Saccharomyces cerevisiae*) encode catabolic proteins and some of these genes may explain the organism's commensal and pathogenic survival.^[83] Other well-known features include genomic diversity resulting from interchangeable cell morphologies, the ability to change the colony phenotype at high frequencies, and a predisposition to "chromosomal shuffling" mechanisms.^[84]

Candida albicans, which lives in harmony with other members of microbiota in people with healthy immune systems, rapidly reproduce and cause infection as a result of balance disorders caused by changes in the local environment

(pH changes or dietary changes), antibiotic use, or changes in the immune system (due to infection or immunosuppressive therapy);^[85-87] these changes induce hyphae formation in response to unfavorable growing conditions or environmental signals that presumably indicate a hostile environment.^[88,89] Many virulence-related traits of pathogenic *Candida* species have evolved to facilitate commensal survival, for example in the fluctuating environment of the gut and to compete with commensal bacteria. Acquired mechanisms to cope with adverse conditions as a commensal organism may also promote virulence, as they equip the fungus with the weapons necessary to overcome host barriers.^[90,91]

Candida albicans forms highly structured biofilms composed of multiple cell types (round budding yeast cells, oval pseudohyphal cells, and long hyphal cells) located in an extracellular matrix.^[93] *Candida albicans* is often isolated from infections related to medical devices, including urinary and central venous catheters, pacemakers, mechanical heart valves, joint prostheses, contact lenses, and dentures

(Figure 4).^[92,94] When a *Candida albicans* biofilm forms on an implanted medical device, it acts as a reservoir for pathogenic cells, is highly resistant to drugs and the host immune system, and has the potential to cause generalized bloodstream infections (candidiasis).^[12] Since these fungal biofilms are largely resistant to known antifungal drugs,^[95] treatment of these infections involves the surgical removal of the colonized medical device in combination with administration of high doses of antifungal agents.^[96] However, the excessive use of these antifungal drugs causes significant complications, severe side effects, higher costs, lower efficiency, as well as the development of multi-drug resistance.^[13]

Methylene blue, which is used in the treatment of many diseases due to its high photostability and easy elimination from the body,^[97] has also been used as an antifungal by applying photodynamic therapy in *Candida albicans* vaginal candidiasis in murine models.^[98,99] One study demonstrated that the antifungal effect of MB is due to

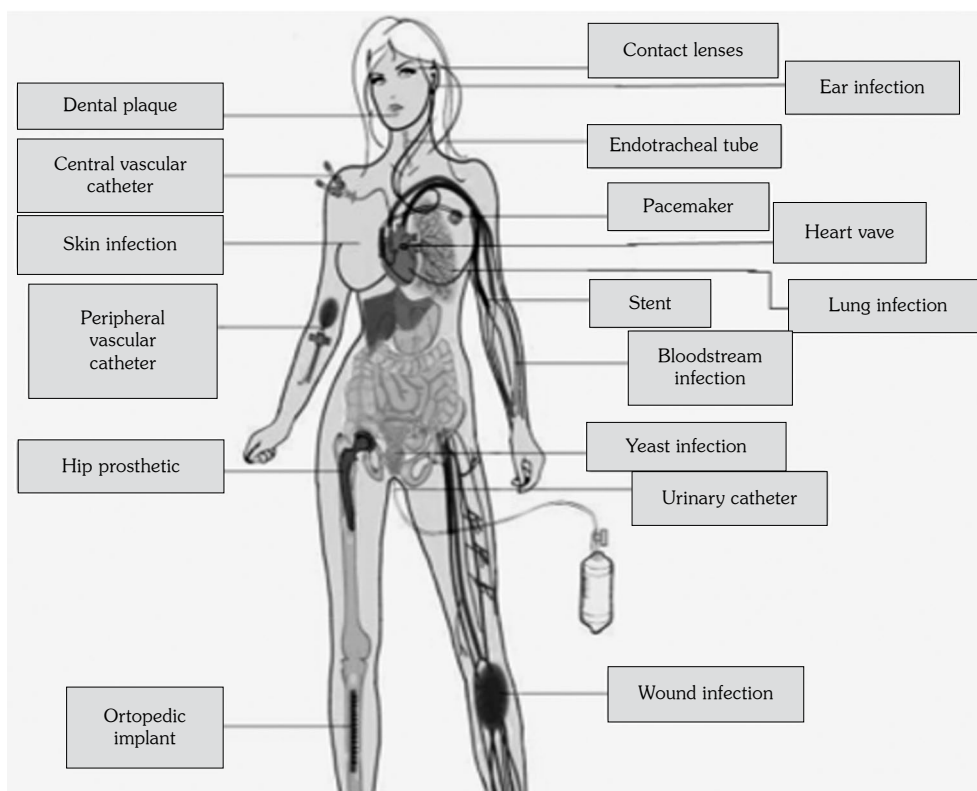


Figure 4. Generalized infections related to *Candida albicans*.^[92]

mitochondrial dysfunction and changes in the redox cycle, as well as membrane homeostasis, which is the target of many antifungals. Yeast-to-hyphae switching is one of the most important factors dictating virulence in *Candida albicans* infections. One study observed that filamentation was completely absent in MB-treated cells and were present only in yeast form, unlike *Candida* cells that were not treated with MB.^[14] We can argue that MB, which is mostly known for its antioxidant properties, can also be used as an antifungal agent to prevent MDR development, based on its ability to cause mitochondrial dysfunction in *Candida*, affecting the redox cycle, and preventing hyphae formation.

DISCUSSION AND CONCLUSION

Methylene blue, an aniline-based thiazine dye that was used for cotton dyeing in the 1800s, has been used in the treatment of many diseases in later years. First proposed by Paul Ehrlich that it could neutralize pathogens, MB was used in the treatment of the malaria epidemic that occurred during World War I and yielded positive results. Later, MB began to be used in multi-drug therapies to amplify its effect. With the discovery of its antioxidant properties, it was proposed as a therapeutic agent in neurodegenerative and psychotic disorders with its features of mitochondrial dysfunction and redox cycle regulation. Methylene blue has been proven to inhibit the accumulation of tau proteins, which is considered among the causes of AD, treat mitochondrial dysfunction with its regulating effect on the redox cycle, and to inhibit formation of ROS molecules. Thus, it has become a very specific agent in the treatment of neurodegenerative disorders, especially AD. Although the *Candida* genus of yeast are commensal organisms, their formation of biofilms on medical devices implanted in the body causes candidiasis infections. With the knowledge of the antioxidant properties of MB, it has been observed to reduce infection by causing mitochondrial dysfunction in yeast. The antifungal property of MB has been demonstrated in candidiasis treatment. Considering that MB is also used in other conditions such as methemoglobinemia, psychiatric disorders, and vasoplegic syndrome, it is evident that MB is a highly multifunctional agent.

Declaration of conflicting interests

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