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Evaluation of Methylene Blue as An Effective Antiseptic for Medicinal Leeches (*Hirudo verbana*)

Metilen Mavisinin Tıbbi Sülükler (Hirudo verbana) için Etkili Bir Antiseptik Olarak Değerlendirilmesi

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ABSTRACT

Objective: Medicinal leeches (*Hirudo* spp.) have been used for therapeutic purposes in humans since ancient times. Because of their growth conditions, leeches carry certain bacteria and endosymbionts (e.g., *Aeromonas* spp). In both leech farms and hirudotherapy clinics, there are no reliable antiseptics that can be used with leeches. This study aimed to determine whether methylene blue (MB) is a safe antiseptic for medicinal leeches and assess its safe usage.

Methods: This study evaluated the efficacy of MB by determining lethal concentrations (LC), effective concentrations (EC), and lethal times (LT) for the medicinal leech *Hirudo verbena* Carena, 1820. A total of 570 *H. verbana* specimens obtained from a local farm were used in this study. Eighteen different concentrations of MB (between 1 ppm and 512 ppm) were tested.

Results: The LC_{50} and EC_{50} values for *H. verbana* were determined to be 60.381 (53.674-66.636) ppm and 2.013 (1.789-2.221) ppm, respectively. The LT_{50} durations for MB concentrations of 32 and 512 ppm were calculated as 212.92 h (138.43 h-1485.78 h) and 17.82 h (8.08 h-23.90 h), respectively.

Conclusion: The results show that MB concentrations between 2 and 19 ppm can be safely used as antiseptics in hirudotherapy clinics and leech farms to address bacterial concerns caused by medicinal leeches.

Keywords: Medicinal leech, hirudotherapy, methylene blue, antiseptics, lethal concentrations, lethal times

ÖZ

Amaç: Tıbbi sülükler (*Hirudo* spp.) eski çağlardan beri insanlarda tedavi amaçlı kullanılmaktadır. Sülükler, büyüme koşulları nedeniyle bazı bakterileri ve endosimbiontları (örneğin; *Aeromonas* spp.) taşırlar. Hem sülük çiftliklerinde hem de hirudoterapi kliniklerinde sülüklerle birlikte kullanılabilecek güvenilir antiseptikler bulunmamaktadır. Bu çalışmanın amacı, metilen mavisinin (MB) tıbbi sülükler için güvenli bir antiseptik olup olmadığını belirlemek ve güvenli kullanımını değerlendirmektir.

Yöntemler: Bu çalışmada, tıbbi sülük Hirudo verbana Carena, 1820 için ölümcül konsantrasyonlar (LC), etkili konsantrasyonlar (EC) ve ölümcül süreler (LT) belirlenerek MB'nin etkinliği değerlendirilmiştir. Bu çalışmada yerel bir çiftlikten elde edilen toplam 570 *H. verbana* örneği kullanılmıştır. On sekiz farklı MB konsantrasyonu (1 ppm ile 512 ppm arasında) test edilmiştir.

Bulgular: *H. verbana* için LC_{50} ve EC_{50} değerleri sırasıyla 60.381 (53.674-66.636) ppm ve 2.013 (1.789-2.221) ppm olarak belirlenmiştir. 32 ve 512 ppm MB konsantrasyonları için LT_{50} süreleri sırasıyla 212.92 saat (138.43 saat-1485.78 saat) ve 17.82 saat (8.08 saat-23.90 saat) olarak hesaplanmıştır.

Sonuç: Sonuçlar, 2 ila 19 ppm arasındaki MB konsantrasyonlarının, tıbbi sülüklerin neden olduğu bakteriyel endişeleri gidermek için hirudoterapi kliniklerinde ve sülük çiftliklerinde antiseptik olarak güvenle kullanılabileceğini göstermektedir.

Anahtar Kelimeler: Tıbbi sülük, hirudoterapi, metilen mavisi, antiseptikler, öldürücü konsantrasyonlar, öldürücü süreler

INTRODUCTION

The therapeutic potential of medicinal leeches has long been recognised, and their saliva contains a potent anticoagulant compound. These natural healing creatures have a rich history of use in various cultures, including Central Asian countries, Egypt, Arabian regions, Anglo-Saxon communities, and ancient Greek and Roman civilisations (1-3). The application of hirudotherapy, which includes the use of medicinal leeches, is among the most popular methods in clinics in France, Russia, Canada, and Germany. These extraordinary creatures can even be found in European pharmacies, and more than 300 hirudotherapy clinics offer medicinal leech treatment

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in Germany alone (4). In particular, in 2004, the US Food and Drug Administration authorised medicinal leeches in plastic and reconstructive surgery. As a result, their adoption in traditional and complementary medicine practices (TCMP) has gained global momentum and led many countries to establish legal frameworks to regulate their use (5-9).

As confirmed by many studies, medicinal leeches require endosymbiont bacteria, especially *Aeromonas* species, to digest the blood they consume (10-12). Unfortunately, medicinal leeches have also been associated with *Aeromonas* infections in patients requiring antibiotic treatment (10,13-17). To address this problem, researchers found that immersion of *Hirudo medicinalis* Linnaeus, 1758 in a 12.5 ppm hypochloric acid solution for 10 min effectively disinfected the handling material and suppressed oral bacterial flora without harming the leeches or compromising their sucking function (18). Furthermore, the antibiotics ciprofloxacin and cefotaxime added to the feeding blood of medicinal leeches significantly reduced the number of *Aeromonas* species (19).

MB is a heterocyclic aromatic compound characterized by the chemical formula $C_{16}H_{18}ClN_3S$ H_2O. This dark greenish-blue dye has a wide range of applications in medicine, biology, and chemistry. It is classified as a thiazine dye because of the presence of nitrogen and sulphur atoms in the aromatic ring structure. It has a molecular weight of 319.85 g/mol, making it highly soluble in water. Its synthesis in 1876 marked the beginning of its widespread use in various fields (20). In 1928, Schultz (21) elucidated the ability of MB to photoinactivate bacteria, leading to its widespread adoption. Later, Perdrau and Todd (22) revealed the antiviral mechanism of action. Notable applications of MB include surgical staining, treatment of malaria, and treatment of methaemoglobinemia. MB effectively treats methaemoglobinemia by reducing methaemoglobin to haemoglobin, which is crucial for oxygen transport in the bloodstream. Furthermore, MB serves as a remedy for cyanide poisoning by detoxifying the body by converting cyanide into less toxic substances (23). Beyond these critical applications, MB also plays a role in photodynamic therapy for lung, breast, and prostate cancers. Remarkably, no cases of severe acute respiratory syndrome-coronavirus-2 infection associated with MB use in chemotherapy patients have been reported in France (24). In addition, low doses of MB have been shown to stimulate mitochondrial respiration *in vivo*, and its safety has been well established in animal and human studies (25). In aquaculture, MB prevents bacterial, parasitic, and fungal diseases in fish. It is widely used as a disinfectant in aquarium fisheries, where it has proven to be particularly effective in preventing fungal diseases (26-29). MB has recently expanded so much that its potential as an anti-aging drug has emerged (30).

Despite sterile farm conditions, leeches still carry bacteria as endosymbionts or are contaminated by soil or water. Consequently, it is important to develop antiseptic solutions that can safely eliminate, prevent, or minimize these bacteria before TCMP. However, the mechanisms of action and LC of many antibiotics and antiseptics used in leeches have not yet been determined. Although medicinal leeches are hardy, they are highly sensitive to water quality criteria and chemicals, emphasizing the importance of establishing safe use ranges for chemical antiseptics to protect leech health, prevent contamination on farms, and avoid harm to leeches. Despite the widespread use of MB as an antiseptic in various medical fields, its toxicity in medicinal leeches, which are widely used in clinical applications, requires further investigation. This study aimed to fill this gap by investigating the acute toxicity of MB on medicinal leeches (*H. verbana*) and determining the safe dose range for its use in both clinics and leech farms. The results of this study will contribute to a broader understanding of the effects of this important compound on different organisms and ensure the safety and efficacy of MB in leech treatment.

METHODS

Medicinal Leeches Used in This Study

Medicinal leeches were obtained from a private medical leech production farm in Kayseri (Türkiye) and transported live to the laboratory for research. The average weight, length, and width of *H. verbana* used in the MB test were 1.49±0.16 0.16 g (1.24-187 g), 89.95±6.42 6.42 mm (79.80-99.30 mm) and 10.30±1.02 mm (9.2-12.1 mm), respectively. Considering that leeches feed on blood, they were given three months to digest this blood and acclimatize to the laboratory environment. During this adaptation period, the aquariums established in the laboratory provided water exchange and oxygen support to the leeches. After the completion of the 3-month adaptation period, toxicity tests (LC_{50}, EC_{50} and LT_{50}) were performed to determine the efficacy of MB on *H. verbana*.

Experimental Design

This study utilized a static acute toxicity assay, as prescribed by the APHA (31) standard method, to determine the LC_{10-90}, EC_{10-90} and LT_{10-90} values of MB ($C_{16}H_{18}ClN_3S$ x H_2O; Sigma-Aldrich) for medicinal leeches (*H. verbana*) throughout 24, 48, 72 and 96 h. In this experiment, nineteen aquariums were used to study the effects of MB on leeches. One aquarium was designated as the control group, while the remaining eighteen were test groups, each containing 20 L of spring water. The water quality characteristics in each aquarium were carefully monitored, with the temperature maintained at 20 °C and dissolved oxygen and pH values falling within the range of 5.00-5.37 ppm and 7.21-7.26, respectively, in accordance with APHA (31) guidelines. A stock solution of MB was created to prepare for the experiment at a daily concentration of 10 g/L. The solution was used to achieve concentrations of 1, 2, 4, 8, 16, 25, 32, 50, 64, 75, 100, 125, 128, 150, 175, 200, 256, and 512 ppm in the eighteen test group aquariums. Aeration was applied to each aquarium for 2 h to ensure that the MB concentration was evenly distributed throughout the water. In contrast, the control group aquarium contained only spring water without any MB solution. Ten medicinal leeches were placed in each aquarium, resulting in 190 leeches per test replication, including the control group. The experiments were performed in three replicates, and 570 medicinal leeches were used. The mortality of leeches in each replicate was monitored 24, 48, 72, and 96 h after the start of the experiment and recorded for evaluation. Dead leeches were immediately removed from the experimental media. The toxicity parameters in the study were obtained on the basis of the data of medicinal leech mortality at each concentration.

Statistical Analysis

The mean, standard deviation, and range values with 95% confidence limits were used to express the results. All replicates were included in the calculation of the mean values. Statistical analysis was performed using the computer program SPSS 25 (SPSS Inc.). Statistical probit analysis was employed to determine the LC_{10-90}, EC_{10-90}, and LT_{10-90} values based on the MB acute toxicity test data. The chi-square test was conducted to compare mean mortality values using a significance level of 0.05. The relationship between the LT data was determined by correlation analysis. The Kruskal-Wallis test was used to statistically analyse the LC, EC, and LT between the concentrations tested with MB for 96 h. ANOVA Dunnett's T3 analysis was performed to determine differences between groups.

RESULTS

Behavioural Changes in Leeches

The present study investigated the effects of exposure to varying concentrations of MB on the mortality and behaviour of medicinal leeches, depending on the exposure of adult *H. verbana*. The number of dead medicinal leeches was examined across a range of MB concentrations (1-512 ppm), and the results were recorded for 96 h. Notably, no deaths were observed in the test groups exposed to MB concentrations between 1 and 25 ppm. No significant behavioural abnormalities were detected in repeated trials conducted with these leeches. However, behavioural responses were observed on the first day of exposure to higher concentrations of MB. The findings indicate that the mortality of medicinal leeches is dose-dependent on MB concentration and exposure duration, whereas behavioural changes were primarily observed at higher MB concentrations. The survival rates of leeches according to MB concentrations within 24-96 h are shown in Figure 1.

Observations revealed that movements such as excessive mucus secretion, acceleration in their actions, and attempts to move away from the test environment were observed in leeches exposed to 32 ppm of MB after 24 h. Additionally, blue staining in the colors of medicinal leeches, loss of adhesion ability on the posterior sucker of leeches and inactivity of some leeches on the water floor were detected at the same concentration. These behaviours and changes were also detected in other MB concentrations, but the severity was higher and occurred in a shorter time. Similar behavioural changes occurred at 50-75 ppm in 24 h, while at 100-512 ppm, it was observed to take shape in the first 3-6 h. Excess mucus secretion in leeches started at 12 h at 50-75 ppm doses and was detected within the first three h at 100-512 ppm concentrations.

Lethal (LC) and Effective (EC) Concentrations

H. verbana, when exposed to increasing concentrations of MB for 96 h, demonstrated a significant increase in the number of deaths ($p<0.05$ per case). The first death in medicinal leeches occurred at the end of 24 h at MB concentrations ranging from 100-512 ppm, and subsequent deaths were observed at concentrations of 50-75 ppm in 48 h and 32 ppm in 96 h. Furthermore, a significant difference in the number of deaths was observed over the four designated time intervals (24, 48, 72, and 96 hours) in leeches exposed to MB concentrations ($p<0.05$; chi-square test). Additionally, significant differences in the number of dead leeches were observed between the 24-96-h period for each concentration ($p<0.05$), with the highest concentration of MB (512 ppm) resulting in the highest leech mortality. The 24, 48, 72, and 96 h LC_{50} values (95% confidence limits) of MB, determined using a static bioassay system for *H. verbana*, were calculated as 223.429 (182.520-301.140) ppm, 129.192 (116.232-143.853) ppm, 84.430 (77.028-91.752) ppm, and 60.381 (53.674-66.636) ppm, respectively. Moreover, a significant difference in LC_{10-90} values was obtained at different times of exposure ($p<0.05$) (Figure 2, see Supplementary Data Table S1). Our results indicate that high concentrations of MB are toxic to *H. verbana*. However, EC_{10-90} used for sterilization and disinfection in leeches were calculated from the LC values obtained in this study, given that MB is used as an antibacterial agent. The EC_{90} (95% confidence limits), including the factor of safety or *H. verbana*, was determined in the range of 24, 48, 72, and 96 h as 18.983 (13.046-40.410) ppm, 10.409 (8.732-13.313) ppm, 5.147 (4.624-5.924) ppm, and 3.816 (3.411-4.417) ppm, respectively (Figure 3, see Supplementary Data Table S2). Our findings demonstrate that MB concentrations within the range of 2-19 ppm can be safely used to sterilize medicinal leeches and disinfect items used in medicinal leech farms and hirudotherapy clinics. Furthermore, the difference between the LC_{10-90} and EC_{10-90} concentrations of MB occurring after 24 h and the concentrations obtained at the end of 72 and 96 h was determined to be significant ($p<0.05$).

Lethal Times (LT)

The LT50, which represents the time for 50% mortality, was determined as 17.82 h (8.08-23.90 h) for the highest MB concentration of 512 ppm. For the lowest concentration of 32 ppm, the LT50 was calculated as 212.92 h (138.43-1485.78 h) (see Supplementary Data Table S3). The LT_{10-90} for *H. verbana* were analysed at various concentrations of MB using correlation analysis, Kruskal-Wallis analysis, and ANOVA-Dunnett T3 analysis. The results are summarised in Figure 4, which presents a statistical difference matrix indicating the significance of differences between the various concentrations of MB. It was determined that there was a strong positive correlation between the LT obtained according to the concentrations of MB (Figure 4A). Overall, the matrix shows that there were significant differences in LT between the different concentrations of MB. However, specifically, concentrations of 32-64 ppm, 75-125 ppm, and 128-512 ppm showed no significant differences in LT among themselves ($p>0.05$), whereas all other concentrations (75 ppm-512 ppm) showed significant differences in LT compared with each other ($p<0.05$). Moreover, the matrix reveals that the LT at

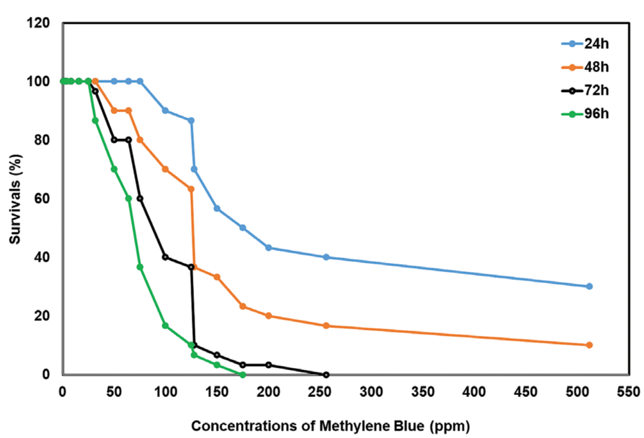

Figure 1. Survival rates of medicinal leeches in the 24-h to 96-h range at MB concentrations

MB: Methylene blue

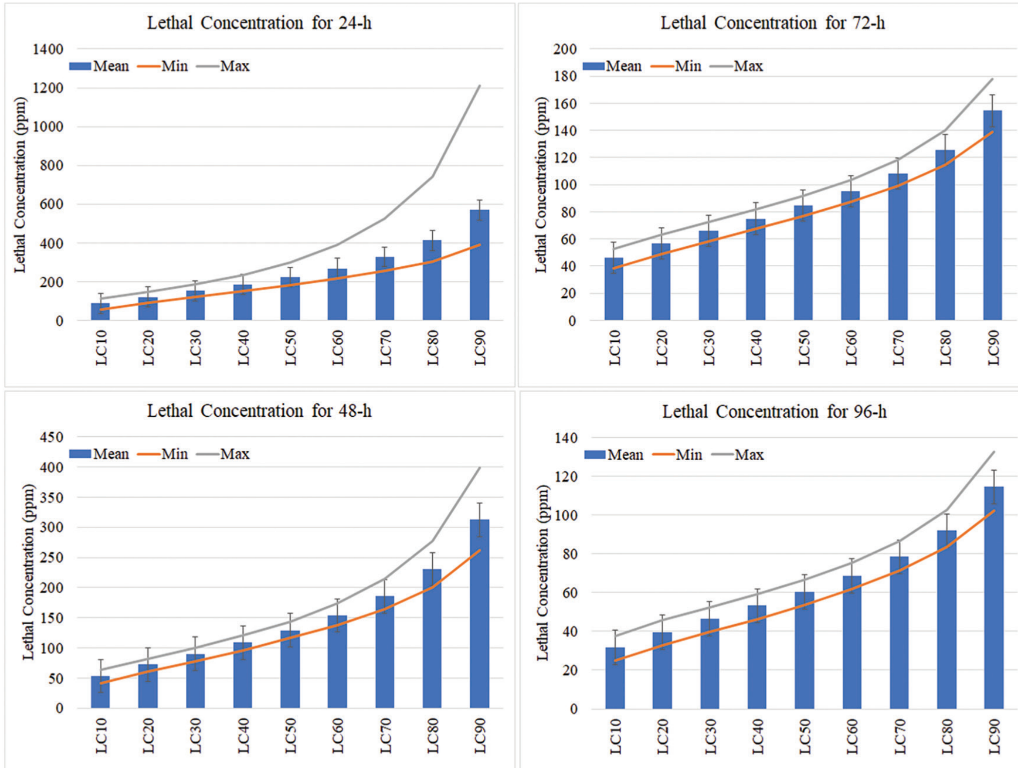

Figure 2. Time-dependent LC_{10-90} of MB for *H. verbana* over 24-96 h (min and max values were obtained within 95% confidence limits)
MB: Methylene blue, LC: Lethal concentrations

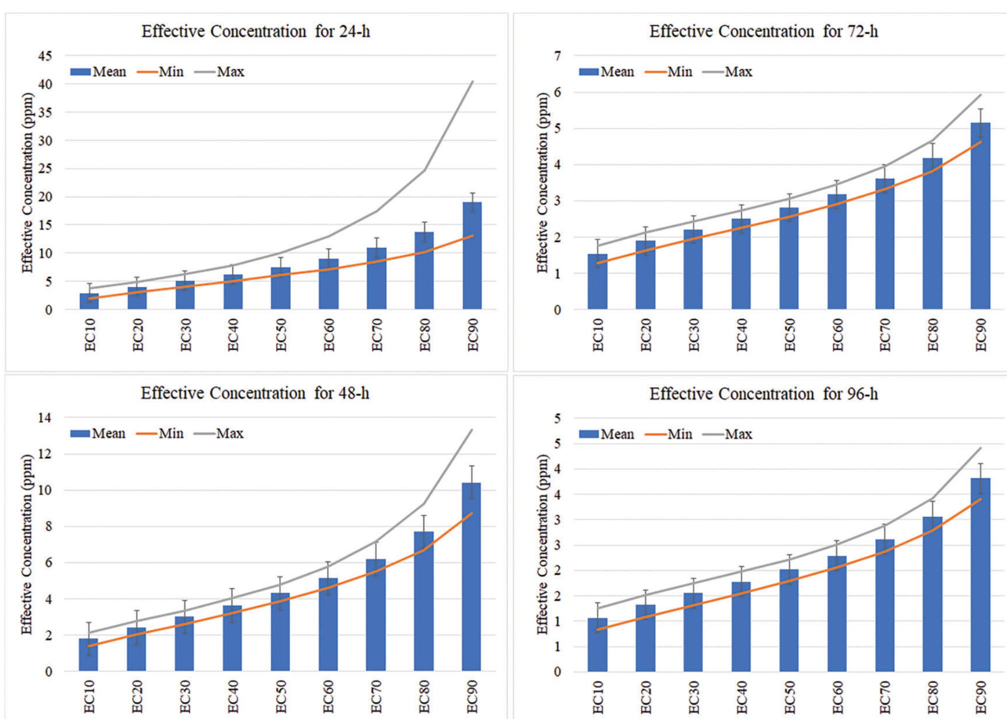

Figure 3. Time-dependent EC_{10-90} of MB for *H. verbana* over 24-96 h (min and max values were obtained within 95% confidence limits)
MB: Methylene blue, EC: Effective concentrations

lower concentrations of MB was generally longer, whereas the LT at higher concentrations was generally shorter. For example, concentrations of 32, 50 and 64 ppm had longer LT, whereas concentrations of 128, 150, 175, 200, 256 and 512 ppm had shorter LT (Figure 4B).

DISCUSSION

This study investigated the toxicity of MB on *H. verbana* and its effect on the survival rate at different concentrations and exposure times. These findings demonstrate that the toxicity of MB on *H. verbana* increased in proportion to both the concentration and exposure time. The medicinal leeches exposed to 32 ppm MB exhibited a mortality rate of only 20% at 96 h, whereas exposure to 512 ppm MB resulted in 100% mortality at 72 h. Moreover, the LC_{50} values of MB for *H. verbana* were determined to be 215.285 ppm after 24 h and 55.271 ppm after 96 h. These results indicate that high concentrations of water-soluble MB can be highly toxic to *H. verbana* if appropriate concentrations are not used.

A study on the toxicity of MB in Nil tilapia [*Oreochromis niloticus* (Linnaeus, 1758)] reported abnormal behaviour, irregular swimming, and premature death in fish exposed to high concentrations of MB. In addition, they determined that the toxicity observed when MB is used in high doses can occur by transferring in the same way (32). These results are consistent with our findings, demonstrating similar effects of MB in leeches exposed to high concentrations. These results agree with our findings showing similar effects of MB in leeches exposed to high concentrations. Again, this reveals that MB can have significant toxic effects on all aquatic living organisms when used in high concentrations.

The LC_{50} values of MB were calculated in three different groups of the marine medaka [*Oryzias dancena* (Hamilton, 1822)], including adults, juveniles, and eggs, after 96 h of exposure as 185.26, 103.84 and 127.15 ppm, respectively (33). In larval fathead minnows [*Pimephales promelas* (Rafinesque, 1820)], the 96-h LC_{50} of MB was found to be 45 ppm at 20 °C (34). Our study determined the LC_{50} value of MB in the medicinal leech *H. verbana* to be 60,381 ppm after 96 h of exposure. These results indicate that *H. verbana* is more sensitive to MB than marine medaka, but more resistant than larval fathead minnows.

In a recent study aimed at determining the lethal dose of zinc in *H. verbana*, it was reported that increasing the concentration of zinc led to behavioural disorders, mucus increase, bleeding, and faster death in the leeches (35). Our study also observed similar anomalous behaviours in leeches exposed to MB. Specifically, the LC_{50} value of MB in *H. verbana* was determined to be 215,285 ppm after 24 h of exposure, whereas the LC_{50} value of zinc in the same species was 48.30 ppm over the same period. This finding indicate that zinc is significantly more harmful to leeches because of its heavy metal nature. At the same time, MB is relatively safe for leeches when used carefully, especially for disinfectant purposes.

Studies have determined the LD_{50} value of copper sulphate on medicinal leeches at low concentrations, specifically at 0.0044 ppm (36). Meanwhile, the LC_{50} value of Cu on *H. verbana* was found to be 0.84 ppm and 15.83 ppm for Zn, indicating a higher sensitivity of *H. verbana* to Cu than to Zn. However, the effect of the mixture of Cu and Zn was higher than that of both elements individually (37). Furthermore, copper has been observed to cause deformations on the body wall of *H. verbana* (38). The mixing of these substances, particularly in the surface waters of natural wetlands, can limit the survival of leeches. Notably, *H. verbana* displays greater resistance to MB solutions than to chemicals such as Cu, Zn, and copper sulphate.

Water-borne heavy metal accumulation in tissues and salivary secretions of *Hirudinaria manillensis* (Lesson, 1842) has been reported in natural environments. However, a study has shown that heavy metal accumulation can decrease by 92.4-99.7% after three weeks of continuous exchange with distilled water (39). This indicates that heavy metal accumulation in *H. verbana* after using MB as a bath can be easily removed when the leeches are transferred to clean water. Moreover, short-term antiseptic applications of MB (e.g., 30 minutes) are unlikely to accumulate in leeches.

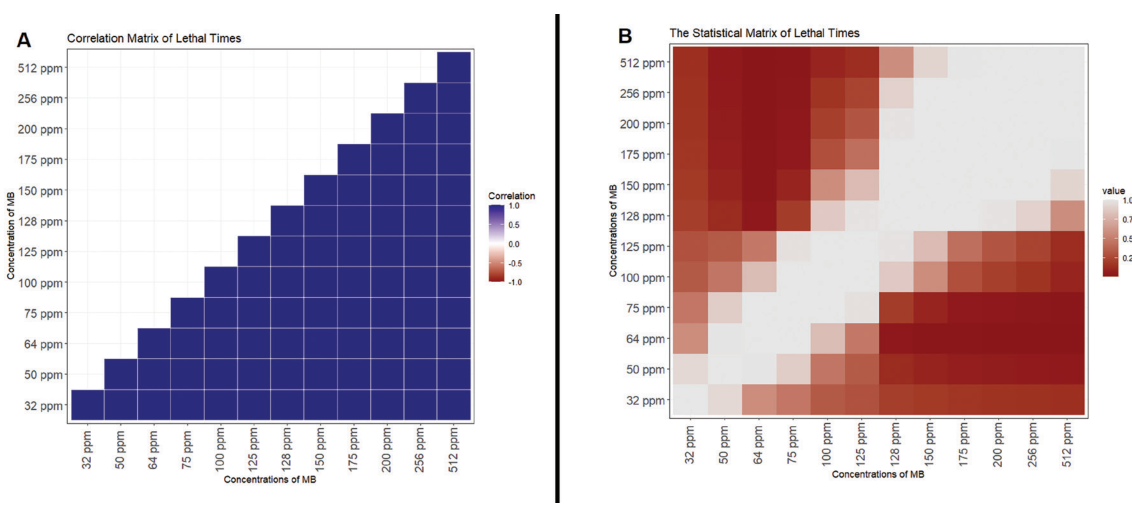

Figure 4. A) Correlation of lethal times obtained with respect to concentrations of MB. B) ANOVA-Dunnett T3 analysis showing the statistical matrix of LT_{10-90} at varying concentrations of MB used for *H. verbana*. (The change of the p-value from grey to dark red from $p<0.05$ to $p>0.05$ indicates)

MB: Methylene blue, LT: Lethal times

A study has suggested that hypochlorous acid, when used at a concentration of 12.5 ppm, can serve as a safe and effective external decontamination and bacterial suppression method for medicinal leeches without causing any adverse effects on their life or sucking function (18). Nonetheless, chlorinated compounds, including hypochlorous acid, are highly toxic to leeches and should be avoided in leech farming practices (40). Additionally, the presence of chlorine compounds can cause leeches to vomit. Therefore, based on our toxicological findings and effective doses, we recommend using MB as a safe and effective alternative to prevent external contamination and suppress pathogenic bacteria in leeches, without causing harm to the animals.

Previous studies have demonstrated that ciprofloxacin and cefotaxime added to food can suppress *Aeromonas* endosymbionts in the intestine of leeches and control bacterial infections (10,19). Similarly, using MB as a bath at appropriate concentrations can also play a crucial role in reducing the prevalence of *Aeromonas* species both on the surfaces and in the digestive tract of medicinal leeches.

Soft tissue infections and complications resulting from *Aeromonas* infections have been observed in some patients after hirudotherapy (14-16). However, the incidence of such infections can be decreased by taking a bath with MB or other antiseptics and disinfectants one week before using medicinal leeches.

CONCLUSION

MB has a long history of use in medicine and is effective and safe in various applications. This study investigated the toxic effects of different concentrations of MB on medicinal leeches and sought to determine its safe and effective use as an antiseptic. The results showed that longer exposure times to MB concentrations significantly increased mortality and behavioural changes in medicinal leeches. It reveals that MB concentrations between 2 and 19 ppm can be safely used in sterilization of medicinal leeches and can be a valuable tool in reducing the risk of bacterial infection in patients receiving leech therapy. This shows that MB can be safely used as an antiseptic in medicinal leech farms and traditional and complementary medicine clinics, which is becoming increasingly widespread worldwide.

* Ethics

Ethics Committee Approval: Animal Experiments Ethics Committees As stated in the Regulation on Procedures and Principles, an Ethics Committee Approval Certificate is required for vertebrate animals. Because medicinal leeches are invertebrates, they are not included in the definition of “experimental animal”. Therefore, an Ethics Committee approval certificate was not obtained for this study.

Informed Consent: This study was conducted in accordance with the EU Directive 2010/63/EU for animal experiments, the National Research Council’s Guide for the Care and Use of Laboratory Animals, and the National Ethic Rules of Türkiye.

* Authorship Contributions

Surgical and Medical Practices: S.D., S.F., N.S., Concept: N.S., Design: S.F., N.S., Data Collection or Processing: S.F., B.K., N.S., Analysis or Interpretation: S.D., N.S., Literature Search: S.D., S.F. B.K., Writing: S.D., N.S.

Conflict of Interest: No conflict of interest was declared by the authors.

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Table S1. Time-dependent LC_{10-90} of MB for *H. verbana* over 24-96 h

Point	Lethal concentration (ppm) (95% confidence limits)			
	24-h	48-h	72-h	96-h
LC_{10}	87.658 (57.090-111.529)	53.448 (42.196-63.323)	46.165 (38.303-52.906)	31.851 (25.007-37.727)
LC_{20}	120.860 (90.308-147.765)	72.362 (60.677-82.641)	56.796 (48.999-63.502)	39.671 (32.683-45.610)
LC_{30}	152.358 (121.755-186.867)	90.030 (78.308-100.821)	65.950 (58.344-72.656)	46.477 (39.541-52.432)
LC_{40}	185.699 (152.125-235.947)	108.508 (96.559-120.506)	74.932 (67.500-81.792)	53.209 (46.396-59.234)
LC_{50}	223.429 (182.520-301.140)	129.192 (116.232-143.853)	84.430 (77.028-91.752)	60.381 (53.674-66.636)
LC_{60}	268.826 (215.221-391.072)	153.819 (138.352-173.661)	95.132 (87.431-103.478)	68.519 (61.773-75.350)
LC_{70}	327.654 (253.816-523.140)	185.388 (164.984-214.630)	108.089 (99.465-118.466)	78.445 (71.274-86.570)
LC_{80}	413.046 (305.309-741.511)	230.654 (200.955-277.441)	125.510 (114.782-139.856)	91.901 (83.418-102.871)
LC_{90}	569.495 (391.380-1212.311)	312.279 (261.974-399.398)	154.412 (138.706-177.705)	114.466 (102.318-132.519)

MB: Methylene blue, LC: Lethal concentrations

Table S2. Time-dependent EC_{10-90} of MB for *H. verbana* over 24-96 h

	Effective concentration (ppm) (95% confidence limits)			
Point	24-h	48-h	72-h	96-h
EC_{10}	2.922 (1.903-3.718)	1.782 (1.407-2.111)	1.539 (1.277-1.764)	1.062 (0.834-1.258)
EC_{20}	4.029 (3.010-4.926)	2.412 (2.023-2.755)	1.893 (1.633-2.117)	1.322 (1.089-1.520)
EC_{30}	5.079 (4.059-6.229)	3.001 (2.610-3.361)	2.198 (1.945-2.422)	1.549 (1.318-1.748)
EC_{40}	6.190 (5.071-7.865)	3.617 (3.219-4.017)	2.498 (2.250-2.726)	1.774 (1.547-1.974)
EC_{50}	7.448 (6.084-10.038)	4.306 (3.874-4.795)	2.814 (2.568-3.058)	2.013 (1.789-2.221)
EC_{60}	8.961 (7.174-13.036)	5.127 (4.612-5.789)	3.171 (2.914-3.449)	2.284 (2.059-2.512)
EC_{70}	10.922 (8.461-17.438)	6.180 (5.499-7.154)	3.603 (3.316-3.949)	2.615 (2.376-2.886)
EC_{80}	13.768 (10.177-24.717)	7.688 (6.699-9.248)	4.184 (3.826-4.662)	3.063 (2.781-3.429)
EC_{90}	18.983 (13.046-40.410)	10.409 (8.732-13.313)	5.147 (4.624-5.924)	3.816 (3.411-4.417)

MB: Methylene blue, EC: Effective concentrations

Table S3. LT_{10-90} for *H. verbana* at different concentrations of MB

Point	Lethal time (95% confidence limits)			
	32 ppm	**50 ppm**	**64 ppm**	**75 ppm**
LT_{10}	67.33h (48.00-80.27h)	53.43h (34.30-65.38h)	55.30h (41.63-63.98h)	41.15h (27.40-49.92h)
LT_{20}	99.97h (83.27-181.27h)	71.62h (55.72-84.52h)	66.52h (54.87-74.97h)	51.68h (39.48-60.78h)
LT_{30}	132.93h (102.28-394.95h)	88.47h (74.23-108.25h)	76.28h (66.97-85.93h)	60.92h (50.35-70.62h)
LT_{40}	169.58h (119.88-781.53h)	105.95h (89.57-141.68h)	85.73h (76.27-96.37h)	70.88h (60.33-82.82h)
LT_{50}	212.92h (138.43-1485.78h)	125.42h (103.60-187.73h)	95.63h (85.73-110.83h)	79.92h (69.48-97.87h)
LT_{60}	267.33h (159.53-2830.47h)	148.45h (118.23-252.70h)	106.67h (95.60-127.48h)	91.12h (78.42-119.58h)
LT_{70}	341.05h (185.43-5647.57h)	177.82h (135.27-347.95h)	119.90h (105.17-150.57h)	104.85h (88.13-148.70h)
LT_{80}	453.52h (220.93-12687.65h)	219.62h (157.60-509.75h)	137.48h (117.57-184.22h)	123.58h (100.18-194.28h)
LT_{90}	673.33h (294.42-39022.12h)	294.35h (193.97-869.30h)	166.20h (136.40-245.12h)	155.18h (118.83-284.48h)
	100 ppm	**125 ppm**	**128 ppm**	**150 ppm**
LT_{10}	27.10h (17.28-34.32h)	24.27h (15.37-31.08h)	15.97h (8.13-20.93h)	11.77h (5.02-32.00h)
LT_{20}	35.37h (25.62-42.68h)	31.78h (22.68-38.68h)	20.15h (12.32-26.23h)	16.87h (9.85-22.58h)
LT_{30}	42.97h (33.67-50.53h)	38.62h (29.78-45.73h)	24.82h (16.72-31.02h)	20.78h (13.33-26.72h)
LT_{40}	50.73h (41.87-59.25h)	45.62h (37.15-53.32h)	29.63h (21.55-36.03h)	24.33h (15.72-30.80h)
LT_{50}	59.27h (50.35-70.83h)	53.28h (44.93-62.58h)	34.98h (27.08-41.82h)	29.12h (20.55-35.83h)
LT_{60}	69.22h (59.27-84.72h)	62.27h (53.27-74.93h)	41.32h (33.57-49.23h)	34.83h (26.53-42.28h)
LT_{70}	81.72h (69.22-105.77h)	73.55h (62.63-92.75h)	49.35h (41.25-60.07h)	42.18h (33.98-51.75h)
LT_{80}	99.27h (81.73-139.27h)	89.37h (74.35-121.17h)	60.77h (50.90-78.02h)	52.82h (43.52-68.42h)
LT_{90}	130.02h (101.57-206.72h)	117.88h (92.82-178.35h)	81.10h (65.65-116.55h)	72.90h (57.78-106.90h)
	175 ppm	**200 ppm**	**256 ppm**	**512 ppm**
LT_{10}	11.45h (4.98-16.77h)	9.57h (3.22-15.07h)	9.92h (3.43-15.23h)	7.87h (1.47-13.47h)
LT_{20}	15.07h (7.78-20.63h)	12.87h (5.38-18.72h)	12.98h (5.53-18.50h)	10.42h (2.67-16.30h)
LT_{30}	18.38h (10.68-24.03h)	15.95h (7.77-21.95h)	15.75h (7.67-21.35h)	12.75h (4.07-18.77h)
LT_{40}	21.78h (13.93-27.52h)	19.17h (10.58-25.27h)	18.58h (10.35-24.23h)	15.17h (5.82-21.22h)
LT_{50}	25.53h (17.77-31.40h)	22.73h (14.05-28.97h)	21.72h (13.47-27.40h)	17.82h (8.08-23.90h)
LT_{60}	29.92h (22.42-36.22h)	26.98h (18.48-33.52h)	25.33h (17.37-31.27h)	20.95h (11.18-27.12h)
LT_{70}	35.43h (28.22-42.97h)	32.42h (24.32-39.92h)	29.92h (22.42-36.62h)	24.92h (15.60-31.47h)
LT_{80}	43.22h (35.67-54.37h)	40.17h (32.18-51.07h)	36.30h (29.12-45.72h)	30.52h (22.17-38.93h)
LT_{90}	56.92h (46.55-79.87h)	54.08h (43.65-78.07h)	47.50h (38.68-67.27h)	40.40h (32.02-58.90h)

MB: Methylene blue, LT: Lethal times