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Ex-situ biofilm mediated approach for bioremediation of selected heavy metals in wastewater of textile industry

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ABSTRACT

Industrialization plays a major role in strengthening the economy of any country. However, these industries directly or indirectly affect the environment. Industrial wastewater discharge has been reported with certain heavy metals such as chromium, lead, cobalt, and others which are a potential hazard to the water bodies and humans as well. Biofilm is an applied method in the fields of bioremediation for reliving this emerging problem and in the efficient removal of heavy metals from wastewater. Biofilms of *Escherichia coli* and *petroleum soil isolated microorganisms* (*PSIM*) were developed at the V bottom of 96 well microtiter plate. The contaminated water sample was collected from the textile industry, Solan, Himachal Pradesh, India. The biofilms were incubated with the industrial water tested for heavy metals assuming the microbes have the potential to assimilate the heavy metals up to 5 mg/mL of concentration. After the incubation for 1–2 weeks, the microorganisms were able to reduce the level of heavy metals present in the samples which was conveyed by biomass comparison of microorganisms in the successive intervals of time. 0.74×10^{10} cells/mL and 0.77×10^{10}cell/mL of *E. coli* and *PSIM* biofilms were able to tolerate the metal toxicity on incubation for 2 weeks at the highest concentration due to the functional group present extracellular polymeric substance which forms complexes with heavy metals. This leads to the fact that these biofilms have assimilated the heavy metals and are potent for the removal of heavy metals from industrial wastewater.

1. INTRODUCTION

Industries generate a large amount of wastewater which normally contains organic and inorganic compounds and certain toxins as well. Industries are expected to treat their wastewater before discharging it into any water bodies as per the law; however, traces remain in the effluent which ultimately drains into the water sources mainly rivers that sometimes can get entered into the main source of drinking water. This water containing traces of heavy metals can cause adverse harm to human health if consumed long period; an aquatic ecosystem of water bodies is also affected which ultimately leads to the loss of normal flora of the particular area. Heavy metals are an important part of most industries and have been efficiently used in textiles, petroleum, tannery, nuclear power stations, and petroleum combustion industries [1]. Epidemiological studies have found that high exposure to heavy metals in drinking water has led to chronic kidney disease, development of neurodegenerative disorders, and neurotoxicity [2,3]. The main or most common heavy metal found is cadmium, zinc, and lead. Heavy metals are extremely toxic and can cause an adverse effect on nerves, bones, and the liver, along with this can even block the vital enzymatic functions of the body [4].

Chromium which is normally present in textile wastewater and even discharged by some industries has an adverse effect on living bodies. Chromium is mostly used in the cement industry for coating (electroplating) and painting. This metal can remain in the sediment of the water if left untreated and discharged into the water streams. It can lead to adverse effects on the human body such as on inhalation in excess it may irritate the nose and even can cause ulcers in the nose and breathing problems such as asthma and coughing. Exposure of this metal on skin can lead to skin ulcers and allergic reactions. Long-term exposure may lead to damage to the liver, kidney circulation, and skin irritations. Lead is the leading initial requirement for the industries which deals with lead electronic battery, fossil fuel burning, and mining [5,6]. For a very high blood level, lead can lead to the high significance of abortions, hypertension, and can even damage the renal tubule [7]. Nickel is widely used in metallurgical processes such as electroplating and nickel-cadmium batteries. Nickel toxicity leads to reproductive toxicity, immune toxicity, and neurotoxicity [8]. To treat this heavy metal polluted water, biofilm formed by selective microbes is capable of up-taking metals and can be used to eliminate heavy metal from the water body to some extent.

Biofilm can be defined as a structural microbiological community living together in their self-producing matrix which can be of carbon

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polymers, namely, polysaccharides and secreted proteins [9,10]. It is clear that the formation of biofilm occurs with some initial steps such as adsorption of some macromolecules (proteins, polysaccharides, and some small molecules such as lipids) on its surface. There are certain factors such as pH, temperature, nutrients, and oxygen that mediate the formation of biofilm. Around 80% of the bacteria are known to form biofilms, Gram-positive and Gram-negative bacteria both have the potential to produce a matrix [10,11]. Microbial biofilms have been reported for both good and bad aspects. Gram-negative biofilms are reported as the causative agents for spoilage in food industries, pathogens for causing infections in the human body, and contaminants of seafood as well as dairy products [12]. On the other hand, microbial biofilms have a positive role in environmental technologies, production of alcohol, organic acids, bioremediation, wastewater treatment, and removal of corrosion [10,13].

Biofilm-mediated bioremediation is the biennial, productive, and safer opportunity for bioremediation with planktonic microorganisms which have a better chance of survival as they produce within the matrix, due to their behavior, the biofilm is the best alternative to use in industrial plants to immobilization and degradation of pollutant [14]. Biofilms are considered a better alternative over the planktonic individual bacteria because of the greater biomass and immobilization properties which further enhance the adhesive and protective properties [15,16]. The higher is the biomass and the enzymatic property, the higher the ability of the biofilm to reduce toxic waste through the process of biosorption and bioaccumulation [17]. A biofilm EPS, that is, extracellular polymeric substance not only provides structural and functional benefits but also directly participate in the complex formation with the heavy metals due to their functional group such as amide, phenol, and hydroxide. [18,19]. Further, some studies show that the microbes incubated with toxic metals to degrade the toxicity of the metals such as chromium and lead have been reported to catalyze the metals in the part of their metabolism to which the toxicity of the metals was also reduced. Cr^{3+} was reduced to Cr^{4+} which is less toxic and lethal to the environment. This states that the microbes carry the potential of degrading the metal toxicity from the environment and have the ability of bioremediation [20]. Therefore, microbial biofilms can be used for the bioremediation and subsequent degradation of heavy metal by assimilation.

2. MATERIALS AND METHODS

2.1. Collection of Wastewater Sample

5 l of wastewater sample were collected from the textile industry in sterile air tight bottle. The industry was situated in, Solan Himachal Pradesh, India. The collected sample was further accessed to detect the presence of heavy metals.

2.2. Physical and Chemical Test for Water Quality

The collected wastewater was analyzed for various physical and chemical properties such as odor threshold, biological oxygen demand (BOD), pH, chemical oxygen demand (COD), total suspended solids (TSS), and total dissolved solids (TDS) [21].

2.3. Test for Chromium (Cr^{3+})

In 10mL of wastewater, few drops of H_2O_2 were added followed by the addition of 6M sodium hydroxide (NaOH) until the solution turned basic, the test tube was heated at 40–60°C to note the color change. The color of the solution changes from blue to yellow which confirms the presence of chromium. 1M of potassium dichromate was prepared in 25 mL of distilled water which was kept as a positive control for chromium detection where negative control was distilled water [21].

2.4. Test for Nickel Detection (Ni^{2+})

In 10 mL of wastewater sample, 6M ammonia (NH_3) was added until the solution turns basic after that few drops of dimethylglyoxime (DMG) was added to the test tube. As the color of the solution changes to reddish pink after the addition of DMG which confirms the presence of nickel. Nickel ammonium sulfate (0.1M) was prepared in 10 mL of water to which 6M of ammonia was added followed by the addition of DMG to the solution which was served as a positive control and 10 mL of distilled water was served as the negative control [22].

2.5. Test for Nitrates

Two–3 drops of concentrated H_2SO_4 with 1 mL of ferrous sulfate were added to the 10 mL of sample for test in a test tube. A brown ring formed indicates the presence of the nitrates in the sample. NH_4NO_3 (1M) was prepared in 10 mL of distilled water which was kept as a positive control for the reference [23].

2.6. Isolation of Microorganisms for Biofilm Formation

The isolation of microbes was carried out from the soil collected from petrol filling stations, near Kharar, Punjab, India. The soil sample was collected near the petrol storage tank; the sample was taken down from 10 cm depth from the surface. The sample was brought to the laboratory in a sterile air tight container. 1 g of soil sample was mixed with 100 mL of distilled water and was serially diluted. Colonies obtained from the spread plate were referred to for the streak plate method. Isolated pure colonies were further used for the suspension culture. The culture was stored in nutrient agar plates in standard refrigerator at 4°C.

Escherichia coli culture available in the laboratory was also streaked on a fresh nutrient agar plate and further suspension culture was prepared of the same.

2.6.1. Bacterial Identification

To characterize the morphological characteristics of the isolated microorganisms, Gram staining was performed for *petroleum soil isolated microorganisms* (*PSIM).*

2.7. Biofilm Formation using 96 Well Microtiter Plate

For the biofilm formation, sterile V bottom 96 well microtiter plate was autoclaved at 121°C for 15–20 min. The microtiter plate was further washed with 70% ethanol and distilled water. The cultures were diluted in 1:100 with nutrient broth. To each well, 100 µl of diluted culture was dispensed into two different rows of the microtiter plate and was covered. The plate was incubated for 72 h at 37°C allowing the microorganisms to form biofilm at the vinyl V bottom of the plate. After successful incubation, the liquid was washed off the plate; three different series of both *E. coli* and *PSIM* biofilms were prepared in different wells and were allowed to form with five different concentrations of nutrient broth (1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL, and 5 mg/mL, respectively) for a week at 34°C [24-26].

2.7.1. Identification of biofilm

To the biofilm well of the microtiter plate, 0.1% crystal violet was dispensed. The plate was left to stain for around 10 min at room temperature. The microtiter plate was agitated and the excess stain was removed. The plate was inverted and vigorously tapped. Further,

the plate was dried using a sterile blotting paper to remove the excess of the liquid. The plate was allowed to air dry. About 100 µL of 30% acetic acid was added to each stained well. The dye was allowed to solubilize by covering and incubating the plate for 10–15 min under room temperature. The content was mixed in each well by pipetting and then 120 µL of crystal violet/acetic acid solution was transferred from each well to separate flat bottom 96-well plate. The optical density (OD) was measured at absorption wavelength of 600 nm using UV-visible spectrophotometer.

2.7.2. Incubation with wastewater

The excess liquid from the biofilms was washed off and the biofilms were washed with autoclaved distilled water. Around 100 µL of different concentrations of textile wastewater was transferred to each microtiter plate to let the microbial biofilm to act on them. To compare the growth of the biofilms in two different rows, different concentrations of wastewater and media were added which were (1) successive concentrations of nutrient media, 50 µL was added to the first row and (2) row consisted of 25 µL of different concentration of sample wastewater with 25 µL of nutrient broth. The biofilms were incubated for up to 2 weeks and on successive periods (72 h, 1 week and 2 week) the O.D. was measured using UV-visible spectrophotometer.

3. RESULTS

BOD of 5 days for the wastewater sample was recorded 84.2 mg/L and COD for the following was 188 mg/L. The TDS of sample water was 550 mg/L whereas the TSS was 45 mg/L. The appearance of the sample compared to normal tap water was turbid and the threshold for the odor was 5.25. The pH of the sample was 6.4; the water sample collected from the industry was tested for the presence of heavy metals by running standard tests. These chemical tests indicated the presence of nickel and chromium as the yellow color was established in case of chromium [Figure 1], reddish color precipitates indicated the presence of nickel [Figure 2], whereas there was no presence of nitrates in the water sample.

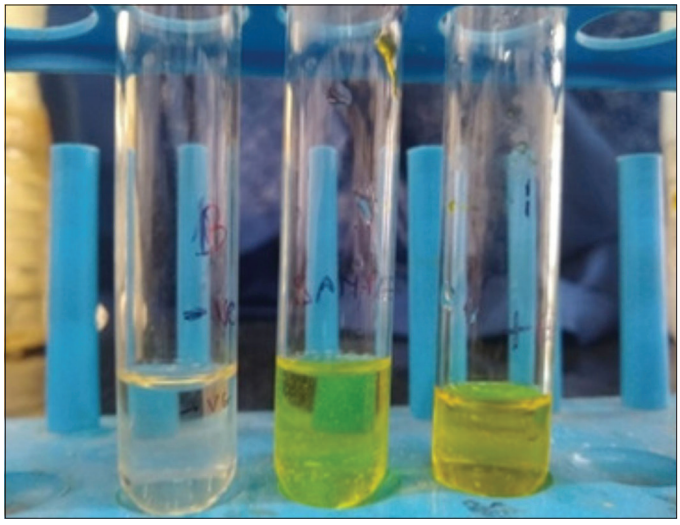

Figure 1: Test tubes indicate test for chromium, first test tube shows the negative control (10 ml of Normal Tap Water), second test tube shows the presence of chromium in sample wastewater, and the third is positive control.

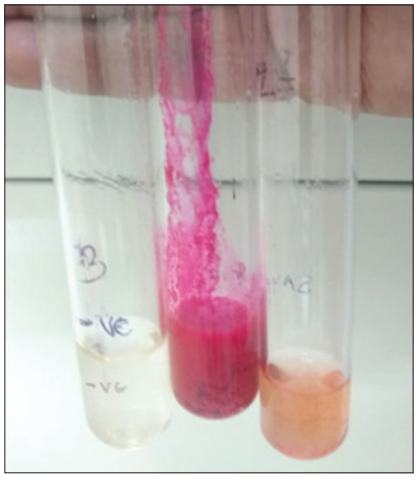

Figure 2: Test tubes indicate the test for nickel, first test tube shows the negative control (10 ml of Normal Tap water), and second test tube shows the presence of nickel in test wastewater sample, and third shows the positive control.

3.1. Characterization of Isolated Microorganism

The colony morphology of *PSIM* was reported as circular distinct colonies with raised elevation. On gram staining, the microbes were reported to retain the crystal violet stain which clearly specified the isolated microbes were Gram-positive, under microscopic observation at ×40 the cells shaped was observed to be spherical in morphology.

3.2. Characterization of Biofilms of *E. coli* and *PSIM*

Allowing the formation of biofilms of *E. coli* and *PSIM* at the V bottom of microtiter plate for 1 week, the initial microbial biomass was recorded by spectrophotometric analysis at A_{600} which is shown in [Table 1]. The cell biomass was calculated by assuming O.D. of 1 that is equal to 8×10^8 cells. A graph between cell biomass and concentration of media shows a gradual increase in cell biomass on increasing the nutrient concentration [Figure 3].

3.2.1. Effect of metal toxicity on E. coli biofilm

Further, after determination of the cell biomass, the biofilms were incubated with different concentrations of nutrient broth and sample wastewater with equal concentrations in combination with nutrient broth. The biofilms were incubated for three successive periods, that is, 72 h, 1 week and 2 weeks. The growth was observed by calculating the cell biomass by measuring the OD at A_{600} to observe the effect of heavy metals on the growth of biofilms. The biomass observed at successive periods for *E. coli* biofilm is shown in Table 2. To determine the effect of heavy metal and to confirm the biofilms has either assimilated the heavy metals or the growth is inhibited, *E. coli* biofilm cell biomass was calculated with reference to the incubation of *E. coli* biofilm under normal environmental conditions to compare the biofilm biomass difference under the observed periods.

3.2.2. Effect of metal toxicity on PSIM biofilm

Similarly, the biofilm of the *PSIM* was incubated with nutrient broth and wastewater as well. The biomass of the microbes was observed in following period, that is, 72 h, 1 week, and 2 weeks. Table 3 shows the cell biomass of the *PSIM* biofilms incubated with nutrient broth as well as wastewater in different concentrations so as to determine the effect of metal toxicity on the growth of *PSIM* biofilm.

Figure 4 shows the comparison between the cell biomass of both the biofilms, where there was an increase in the biofilm biomass of *PSIM* after 2 weeks incubation under high concentrations of heavy metals as compared to the *E. coli* biofilm.

4. DISCUSSION

The experimental study concludes that the wastewater collected from effluent of textile industry was intoxicated with certain heavy metals which were taken into consideration for, namely, chromium and nickel, identified using certain chemical tests. Subsequently, the microbes isolated from petroleum soil sample were characterized through gram staining; the most of the microbes were found to be Gram-positive as they retained crystal violet stain under microscopic observation at ×40 which was further streaked for pure culture. Therefore, the isolated pure colonies were taken for the suspension culture to form biofilms at the bottom of the microtiter plate. Similarly, *E. coli* biofilm was prepared and characterized using 1% CV. In the 1st week of biofilm formation under normal conditions, *PSIM* biofilm showed higher growth than *E. coli*. On incubation of the biofilms with the industrial wastewater, *E. coli* biofilm growth was inhibited at higher concentrations from 3 mg/mL to 5 mg/mL [27]. However, the increase in cell biomass was reported for up to 2 weeks of observation. Similar observations were reported in the biofilms of mixed culture of *Bacillus vallismortis*, *Bacillus haynesii*, and *Alcaligenes aquatilis* isolated from tannery industry which were subjected to variable environment containing chromium, the reduction of Cr (VI) was observed as well as increase in biofilm structure, hot spring isolated that Gram-positive bacteria were also found to tolerate Cr and Cu at different concentrations, similar observations were reported in our findings [28,29]. Most of

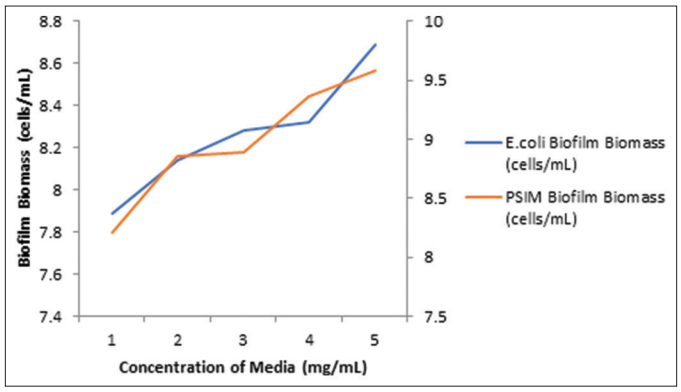

Figure 3: The concentration of media versus biofilm biomass plot shows the *Escherichia coli* biofilm exhibited greater number of cell (cells×10^8) than the PSIM biofilm when incubated with different concentrations of nutrient media.

Table 1: The biomass of the biofilms of both the microorganism was calculated after 1 week of incubation where the biofilm started to develop at the V bottom of the microtiter plate.

Concentration of media used (mg/mL)	*O.D. at A_{600} (E. coli)*	*E. coli biofilm* biomass (cells/mL)	*O.D. at A_{600} PSIM*	*PSIM* biofilm biomass (cells/mL)
1 mg/mL	0.493	3.94×10^8	0.513	4.1×10^8
2 mg/mL	0.509	4.07×10^8	0.553	4.43×10^8
3 mg/mL	0.51	4.14×10^8	0.555	4.44×10^8
4 mg/mL	0.52	4.16×10^8	0.585	4.68×10^8
5 mg/mL	0.543	4.34×10^8	0.5985	4.79×10^8

Table 2: *Escherichia coli* biofilm incubated with nutrient media as well as with wastewater, the biomass was observed after successive intervals i.e., 72 h, 1week and 2-week period. (10^{10} represents the tenth dilution).

	WASTEWATER					
Concentration of Media/ Concentration of Sample Wastewater (mg/mL)	Cell biomass after 72 h of incubation (cells/mL)	Cell biomass after 1 week of incubation (cells/mL)	Cell biomass after 2 weeks of incubation (cells/mL)	Cell biomass after 72 h of incubation (cells/mL)	Cell biomass after 1 week of incubation (cells/mL)	Cell biomass after 2 weeks of incubation (cells/mL)
1 mg/mL	0.48×10^{10}	0.45×10^{10}	0.46×10^{10}	0.32×10^{10}	0.63×10^{10}	0.57×10^{10}
2 mg/mL	0.52×10^{10}	0.68×10^{10}	0.55×10^{10}	0.4×10^{10}	0.68×10^{10}	0.75×10^{10}
3 mg/mL	0.56×10^{10}	0.56×10^{10}	0.56×10^{10}	0.45×10^{10}	0.74×10^{10}	0.61×10^{10}
4 mg/mL	0.56×10^{10}	0.56×10^{10}	0.52×10^{10}	0.422×10^{10}	0.76×10^{10}	0.73×10^{10}
5 mg/mL	0.59×10^{10}	0.57×10^{10}	0.53×10^{10}	0.42×10^{10}	0.76×10^{10}	0.74×10^{10}

Table 3: PSIM biofilm incubated with nutrient media as well as with wastewater, the biomass was observed after successive intervals, that is, 72 h, 1 week, and 2 week period. (10^{10} represents the tenth dilution).

	WASTEWATER					
Concentration of Media/Concentration of Sample Wastewater (mg/mL)	Cell biomass after 72 h of incubation (cells/mL)	Cell biomass after 1 week of incubation (cells/mL)	Cell biomass after 2 weeks of incubation (cells/mL)	Cell biomass after 72 h of incubation (cells/mL)	Cell biomass after 1 week of incubation (cells/mL)	Cell biomass after 2 weeks of incubation (cells/mL)
1 mg/mL	0.46×10^{10}	0.844×10^{10}	0.75×10^{10}	0.45×10^{10}	0.69×10^{10}	0.59×10^{10}
2 mg/mL	0.56×10^{10}	0.89×10^{10}	0.84×10^{10}	0.46×10^{10}	0.72×10^{10}	0.68×10^{10}
3 mg/mL	0.52×10^{10}	0.91×10^{10}	0.91×10^{10}	0.42×10^{10}	0.73×10^{10}	0.76×10^{10}
4 mg/mL	0.56×10^{10}	0.94×10^{10}	0.96×10^{10}	0.48×10^{10}	0.74×10^{10}	0.76×10^{10}
5 mg/mL	0.57×10^{10}	0.97×10^{10}	0.97×10^{10}	0.52×10^{10}	0.88×10^{10}	0.77×10^{10}

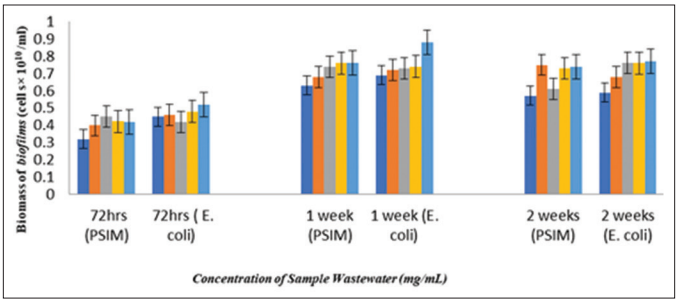

Figure 4: Comparison of cell biomass between the *Escherichia coli* biofilm and PSIM biofilm, where the PSIM showed relative higher growth with increasing period of incubation. The figure shows different dilution of wastewater incubated at different respective periods mentioned above.

the authors have reported immobilization and absorption of heavy metals by the EPS of *Bacilli* biofilm matrix as the principle behind this process of bioremediation [27,30]. The tolerance of Gram-negative bacteria *Rhizobium* toward Cr and Cd showed similar results as of our study where the microbes were able to tolerate the concentrations of 0.1 mg/mL and 1 mg/mL of heavy metals [31]. Similarly, in our study, *PSIM* biofilm possessed a higher ability to form EPS and to absorb the heavy metal with greater resistance, considering our Gram-positive isolated microorganisms have a great potential in the removal of toxic heavy metals than the previously reported studies.

5. CONCLUSION

The experimental procedure leads to the fact that the microbial biofilms of *E. coli* and petroleum isolated microorganisms can be utilized for the degradation of heavy metals present in wastewater which are a threat to the environment. The biofilms of *E. coli* and *PSIM* have somehow resisted the heavy metal load because of the EPS ability to immobilize and absorb the heavy metals forming a stable complex and decreasing the metal toxicity in the environment. *E. coli* has the ability to resist heavy metals such as chromium which reduces Cr (IV) to Cr (III). Similarly, nickel is also required for certain cellular process in microbes but at higher concentration, the growth was somehow inhibited compared to *PSIM* biofilms. This can lead to the fact that the isolated microorganisms assimilate the heavy metals with greater efficiency than *E. coli*. The growth of these biofilms on incubation with industrial wastewater for successive periods has relatively showed the ability of EPS to chelate the heavy metals, namely, chromium and nickel from the industrial wastewater and formed a stable complex. Thus, biofilms of microbes, namely, Gram-positive and Gram-negative (*E. coli*, *Enterococcus*, and *Pseudomonas*) can be a remedial measure to minimize the heavy metal pollution in the water.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

The data used to support the findings of this study are included within the article.

12. PUBLISHER'S NOTE

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