Research Article

Eco-potential of *Aspergillus penicillioides* (F12): bioremediation and antibacterial activity

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Abstract
A total of 112 soil-inhabiting fungal isolates were recorded from different stretches of Subarnarekha river basin (21° 33′ to 23° 32′ north latitude and 85° 9′ to 87° 27′ east longitude), mainly its stretch within the state of West Bengal, India. All isolated fungal groups were grown into potato dextrose agar (PDA) media. Fungal extracts were collected from the grown-up fungus colony after incubation of fungal culture in a liquid medium. Tests for estimation of coliform bacteria were conducted by MPN and MFT methods. Heavy metals were estimated by atomic absorption spectrophotometer, and experimental designs were made following standard literature to observe antibacterial activity as well as to assess heavy metals removing potential of isolated fungal strains. The highest coliform count in MPN test (> 2400 MPN/100 ml) was recorded at S-II followed by S-I and S-III during monsoon season. Out of five identified fungal species (*Fusarium* sp., *Rhizopus* sp., *Penicillium* sp., *Aspergillus* sp. *Pythium* sp.), the fungal strain, *Aspergillus penicillioides* F12 (MN210327) has exhibited the highest heavy metal tolerance activity. It showed resistance against Pb(II) and Cd(II) up to 1000 ppm and Hg(II) up to 200 ppm. Alongside, the specific fungal extracts of this species have also revealed antibacterial activity by proving their effectiveness as potential inhibitor against human pathogenic gram-negative bacteria, *Escherichia coli* and *Vibrio cholerae*, and gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*.

Keywords Heavy metal · *Aspergillus penicillioides* · Coliform bacteria · Antimicrobial activity

Abbreviations

PDA	Potato dextrose agar
MPN	Most probable number
MFT	Membrane filtration technique
Cfu	Colony forming units
SEM	Scanning electron microscopy
EDAX	Energy dispersive X-ray analysis

1 Introduction

Water, being the most essential natural component of the earth, is distributed mostly as sea water (97%) and the remaining (3%) occur as freshwater [1]. More than 80% population in the present world are suffering from freshwater scarcity [2]. Moreover, surface freshwater present in the water bodies like rivers, estuaries, and channels are being continuously polluted by anthropogenic activities such as urbanization, agriculture, and industries. [3]. Heavy metals (arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, silver, zinc, etc.) representing the persistent toxic chemical elements, in nature after being released from several human activities (mining activities, the metallurgical industry, sewage, irrigation and agricultural wastes, pesticides and fertilizers) enter into the soil and water of the riverine system causing several human health problems worldwide [4] by not only acting as cytotoxic but also playing their roles as mutagenic and even carcinogenic pollutants [5]. On the other hand, waterborne pathogen-mediated human diseases are a major water quality concern for human beings especially in the developing countries which have posed serious threats to the

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human survivability mainly because of lack of sanitation system, improper disposal of waste materials, and scarcity of potable water supply [6]. Heavy metals after being accumulated and magnified in many aquatic organisms [7–9] severely affect soil subsystem from where they are transported to upper trophic levels by food chain–food web processes [10]. Alongside heavy metals, biological oxygen demand (BOD) and chemical oxygen demand (COD) have been increased by an intricate interaction among bacterial populations with several other pollutants, both organic and inorganic in nature after entering the river ecosystem such as wastes from the paper mills, sewage from the food and pharmaceutical industries, from the leakages and drains of the septic tanks, pesticides from agricultural runoff, and heavy metals from mining and metal industries [11].

Microorganisms in the polluted riverine flows have shown to manifest different strategies to cope up with deteriorating ecological condition and also to survive in the ecologically stressed environment by adopting different detoxifying mechanisms such as bio-sorption, bioaccumulation, bio-transformation, and bio-mineralization [12]. The scientific principles behind such adaptive strategies and mechanisms of microorganisms can be manipulated and exploited for bioremediation either by ex situ or in situ mechanisms [13, 14]. Microbial biomass of surface soil in the benthic zone of river ecosystem is constituted by about 90% of bacteria and fungi, which tend to play decisive and regulatory roles in nutrient cycling [15, 16]. The previous studies have revealed the higher abundance of fungi in coarse sand fractions, whereas bacteria were recorded in higher densities in silt- and clay-dominated fractions of riverine sediment [17–19].

Waterborne pathogens have been recorded in increasing abundance in coastal-estuarine environment, which tend to pose serious threats to public health [20]. However, fecal coliforms, such as *E. coli,* were found both in bottom sediment and flowing water of river [21]. Higher abundances of pathogens in estuarine ecosystem are supposed to be due to the increased human activities, such as water transportation, and substantial recreation during tourisms [22, 23]. Rhodes and Katorin 1990 identified several pathogens in estuaries such as *Vibrio cholerae*, *Giardia* sp., *Cryptosporidium* sp., *Salmonella* sp., and *Campylobacter* sp. [24].

Many conventional methods (adsorption, photocatalytic degradation, dialysis, coagulation, and filtration) were used for bioremediation of heavy metals [25]. Microorganisms (fungi, bacteria, and algae) provide a good option for remediation of heavy metals, dyes, and other contaminants from wastewater [26–28]. Various fungal genera such as *Penicillium sp*, *Aspergillus* sp., and *Rhizopus* sp. have been used as potential microbial agents to remove of heavy metals from aqueous solutions [29]. Xiao et al. [30] reported a novel technology, with the use of hyper accumulator plants which had proved to be more efficient and convenient method in contrast to the existing traditional ones for obtaining highly efficient bio-sorbents from endophytes. Generally, bacterial contamination is decreased by addition of antibiotics [31] but earlier report stated that some of bacteria identified from the bottom sediments of Indian rivers can resist broad-spectrum antibiotics [32]. In such context, a cost-effective but eco-sustainable alternative wastewater management program is certainly the need of the hour, not only for the human health but also to ensure the survivability of different biodiversity components of riverine ecosystem including fish.

Myco-remediation is an emerging economically viable and eco-sustainable in situ bioremediation technology that uses fungi to eliminate heavy metals from water [33, 34]. It is possible that fungi, living in an aquatic system in association with multi-resistant bacteria, have been pushed to rely on mechanisms other than the endogenous production of the known classes of antibiotics. Earlier reports have shown that more than 180 species of *Aspergillus* could exhibit antibacterial activities [35].

The present research study has attempted to unearth research information pertaining to the potential of selected fungi for the bioremediation of selected heavy metals (Pb, Cd, and Hg) and also to understand their antibacterial roles against various pathogenic bacteria in order to ensure bacteria-free, safe and healthy adequate water supply to human beings by the way of myco-remediation. Major emphasis in this study was laid on ensuring the sustainability of water availability in terms of portability, adequacy, convenience, affordability, and equity.

2 Materials and methods

2.1 Selection of study sites

The Subarnarekha river basin, located between 21° 33′ to 23° 32′ north latitude and 85° 9′ to 87° 27′ east longitude, covers the geographical area (0.6%) of India. The total annual yield of water flowing within the river basins is about 7940 mm^2 [36]. It is a transboundary river with both freshwater and estuarine influences and flowing through three states of India (Jharkhand, Odisha, and West Bengal) after originating from the Nagri, at Ranchi, the state capital of Jharkhand, and ending to the Bay of Bengal at Talsari, the state of Odisha, India. The river basin is studded with a large number of mineral-based industries and mines. The first study site at Muri (S-I) in the upstream, the second study site, Sonakonia (S-II) in the middle stretch and the

third study site, Talsari (S-III) at extreme downstream of this river were selected for the present research study.

2.2 Microbiological analysis of river water

Bacterial load of water was measured by standard plate count (SPC), membrane filtration technique (MFT), and most probable number (MPN) methods. The tests were performed within 24 h of sample collection. The total bacteria were determined by SPC method using nutrient agar plate. The plates were incubated at 37 °C for 24 h, and the total number of colonies was expressed by colony forming units per milliliter (cfu/ml) water sample [37]. The membrane filters provide a rapid and useful means of sampling from water. Such filters are also used for viable counting by laying on a suitable agar plate and allowing to form colonies. Acetate cellulose-type membrane filter (0.45 µm) was used for the detection of total viable bacteria using membrane filtration technique [38]. The MPN method was used to determine the presence of gas-producing lactose fermenters and most probable number of coliforms present in 100 ml of water. The standard MPN method (15 multiple tube dilution technique) was used for the detection of total coliforms by inoculation of samples into tubes of lactose broth (LB) and incubation at 37 ± 1 °C for 48 h.

2.3 Isolation and identification of heavy metal resistance fungi

The soil samples were collected and stored in 4 °C for further experiment. The fungi were isolated from hydrate soil by serial dilution method using PDA containing 200 ppm of Pb, Hg, and Cd individually. The stock solution was made by double distilled water using $Pb(NO_3)_2$, $CdSO_4$, and $HgCl_2$. 200 ppm heavy metal containing PDA medium (25 ml) was poured in sterilized petri dish. After that soil sample was spread and incubated at 28 °C for 2–4 days. After incubation, predominant genera of fungi were collected and purified by pure culture method [39].

2.4 Removal of heavy metals by fungal isolate from liquid media

After observing growth in PDA media, the most tolerant fungal isolates were cultured in PD broth (50 ml) containing 50 ppm concentration of each of different heavy metals (Pb, Cd, and Hg) separately in triplicates. All conical flasks (250 ml) were kept in the shaker at 120 rpm at 28 °C for 7 days. The controlled flask contains 50 ppm of heavy metal and PDB. After filtration, the mycelium was dried in hot air oven at 80 °C for 24 h. Dried fungal biomass was then digested by nitric acid (HNO_3) and per chloric acid ($HclO_4$) at the ratio of 3:1, and the total metal content was measured by atomic absorption spectrometry (UV-1601, Shimadzu). Blank and standard solutions for calibration were used to measure the concentration of heavy metals using a typical set of standard calibration curves with good linear regression [34].

2.5 Study of scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDAX)

Heavy metal-treated fungus was dehydrated by acetone for 20 min. Fungal sample was then put in an E5200 Auto-sputter coater (UK) under vacuum for 15 min. At 20 kV accelerating voltage, SEM image was taken [34]. After measuring the heavy metal absorption, the fungi were dried at 40 °C in an oven. For X-ray dispersion analysis, the dried fungi were placed onto graphite stub [40].

2.6 Extraction of fungal metabolites

Crude extracts of endophytic fungi were prepared as described by Wang et al. [41] with slight modifications. Endophytic cultures were filtered to separate the culture broth and mycelia using filter paper. All filtrates were then added to 95% ethanol with fully stirring and left overnight. Further, the filtrate was concentrated in a rotary vacuum to remove organic solvents and was then dried by freeze drying. The sterile distilled water was then added to powder extract so as to make a concentration of 10 mg/ml which subsequently was sterilized through a Millipore (0.22 µm) for the assessment of antimicrobial activity.

2.7 Determination of antibacterial activity of the fungal extract

Antimicrobial activity of the secondary metabolites from isolated fungi was carried out by the well diffusion method against two gram-negative bacteria such as *E. coli* and *Vibrio cholerae*, and two gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*. [42]. Test bacterial solution of 0.2 ml was evenly spread in sterile Luria–Bertani (LB) broth agar. Then, the fungal metabolite (50 µl) was poured on the inoculated agar plates, but one of them contained no extract. They were incubated at 37 °C for 24 h in culture incubator. After incubation, the diameter of each inhibition zone was measured with millimeter scale. All experimental assessments were conducted in triplicate.

3 Results and discussion

3.1 Sources of pollution in Subarnarekha River

The non-judicious exploitation of natural resources of Subarnarekha river consisting of both living (fishes/molasses)

and nonliving (sand granules) components has proved to be a real threats to the river basin. Basically, unplanned and unregulated dumping of wastes and development of mining and mineral processing industries mostly at the upstream (mainly Ghatsila and Muri.) of this river have been contributing for causing the environmental degradation of the river basin during the last few decades [43]. During the monsoon seasons, the suspended solids and heavy metal loads in the river water are increased due to the erosion through land runoff and transportation of wastes from different industries, exposed solid waste dumping sites and mining activities. Several environmental problems in the vast stretches of river basin have been resulted for the mining of granites, basalts, quartzite, dolerite, sandstone, limestone, dolomite, gravels, and river sands [44]. Besides that, the domestic and industrial wastewater generated from the urban areas, along the stretch of the river after being discharged into the river pose serious pollution threats to riverine flows in the river [45]. Pathogenic bacteria are being introduced into waters in various ways, including leaking of septic tanks, sewer malfunction contaminated storm drains, runoff from animal feedlots, human fecal discharge, etc. [46].

3.2 Coliform count by MPN and MFT method

The results after the bacteriological examination of water samples collected from the three study sites (S-I, S-II, and S-III) of Subarnarekha river are given in Table 1. According to guidelines of World Health organization (WHO), the permissible limit of drinking water is zero coliform/100 ml [37]. The total coliforms in all samples collected from the all study sites in different seasons were found to range from 540 MPN at S-I during premonsoon to > 2400 MPN at S-II during monsoon (Fig. 1). The considerable amount of coliform has been grown in all three study sites during all seasons of 2017–2018.

The highest coliform count in MPN test (> 2400 MPN/100 ml) was recorded at S-II followed by S-I and S-III during monsoon season. At premonsoon, the S- III showed lesser coliform count in comparison with the S-II and S-I (Table 1). The MPN study of water sample is shown in Fig. 1. The presence of > 10 coliforms/dl in water is designated as polluted or unhealthy for drinking purpose [38]. High MPN values in all the samples clearly indicate that the water is highly contaminated with coliform bacteria. In this study, total coliforms are found to be excessively high compared to the WHO and BIS guidelines. Total coliform test resulted in growth of coliform bacteria at a temperature of 37 °C. Coliforms that produce acid and gas from lactose at 42.5 ± 0.2 °C within 24 ± 2 h are also known as fecal coliforms due to their roles as fecal indicators. On a global scale, water contamination by coliform is a major cause of morbidity and mortality, especially in children. As indicated earlier, coliform are acquired directly or indirectly from a human or animal carriers. Risks from drinking water, therefore, only enunciate following the fecal contamination of the supplied water [47].

3.3 Fungus with potential heavy metal removing activity

A total of 112 fungal isolates have been successfully isolated and cultured from the water/sediments collected from three sampling sites of Subarnarekha river. Fungal isolates were then successfully screened against lead, cadmium, and mercury. From the preliminary screening, there were 16 fungi which showed different resistance patterns against at least five of the three chosen heavy metals. Major fungal isolates were closely related to *Aspergillus* sp.

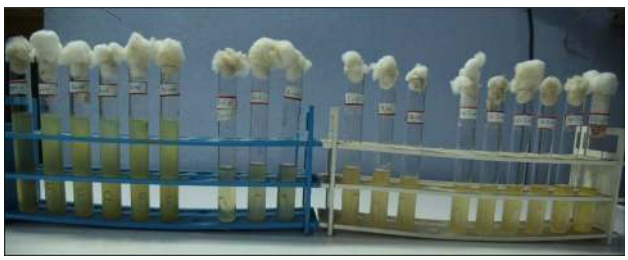

Fig. 1 MPN test at premonsoon season in S-I site of Subarnarekha river water

Table 1 Coliform count in different seasons at different sides of Subarnarekha river

Place	Coliform count	Premonsoon March–June Mean	Monson June–October Mean	Post monsoon November–February Mean
Muri	MPN	540	1600	920
	MFT	520	1340	860
Sonakonia	MPN	1600	> 2400	1600
	MFT	1424	2200	1476
Talsari	MPN	350	920	540
	MFT	254	768	549

F12 (MN210327) (Fig. 2). It has been observed from previous studies that *Aspergillus penicillioides* an aquatic fungus had been isolated from soil sediment of Talsari [34]. All five isolates showed resistance against Pb(II) and Cd(II) up to 1000 ppm and Hg(II) up to 200 ppm. Basically, living organism can absorb metal ions by two ways, one is metabolism-independent way, where cell wall bound to metals ions; and another is intracellular metabolism-dependent pathway, where cell membrane can transport metal ions slowly [48].

Cadmium is considered to be more lethal in later phase of life because of the increasing risk after exposure [49]. Higher concentration of Cd(II) was mainly due to the discharge of effluents from steel industries as well as household discharges, Mercury, being one of the global pollutants, has the ability to move a long distance away from the source [50]. The maximum concentration of Hg was found in the coal-based power plant [51], which can affect the human immune system [52] and cellular disruption [53]. Lead is a neurotoxic and nephrotoxic pollutant and comes into the Subarnarekha river water by the waste of industrial effluents [54].

Among all isolates, *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Rhizopus* sp., and *Pythium* sp. have shown heavy metal scavenging potential. Out of them, *Aspergillus penicillioides* F12 (MN210327) has the highest heavy metal (Pb, Cd) scavenging ability in an optimum P^H, temperature, and time [34]. The scavenging activity of heavy metals (Pb, Cd and Hg) at optimum conditions by *Aspergillus penicillioides* F12 (MN210327) is shown in Fig. 3a, b.

3.4 SEM and EDEX analysis

For SEM study, fungi sample was grown in equal concentration of heavy metal (Pb, Cd, and Hg) containing media. The SEM study revealed that the characteristic of the genus *Aspergillus* sp. F12 (MN210327) is the spore-like bearing structure (Fig. 4a) which produce extracellular polymeric substances (Fig. 4b). The conidia are produced on

Fig. 2 Morphological structure of F12 strain under light microscope

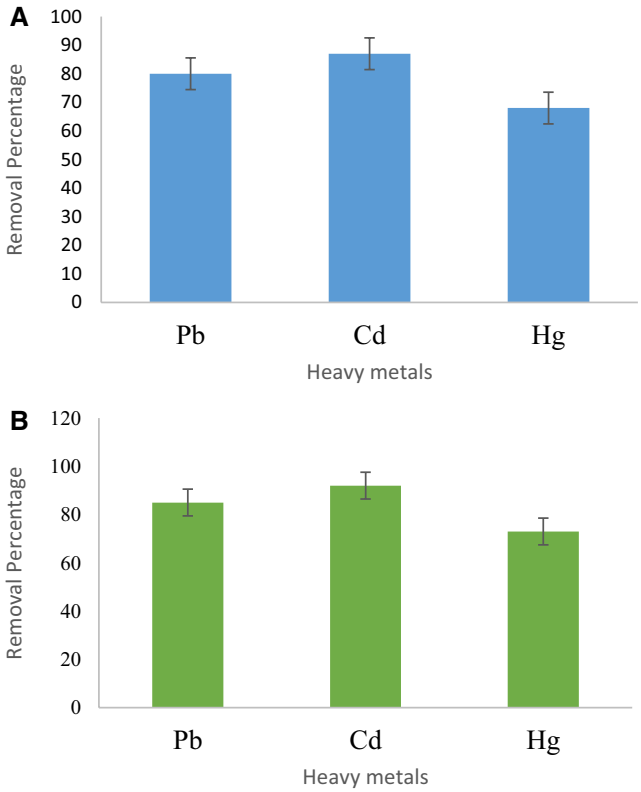

Fig. 3 Percentage of heavy metals removal by *Aspergillus Penicillioides* (F-12) biomass (**a**) and EPS (**b**)

conidiophores arising from the foot cells of the hyaline and septate somatic hyphae. The hypha is branched and multinucleate. The EDEX study of the dry mass revealed the accumulation of target metals in the surface of fungal cell (Fig. 5). The maximum metal accumulation was observed in biomass of fungi, whereas almost double percentage of Pb and Cd has been removed than Hg when supplemented combined with equal concentration.

3.5 Antibacterial activity of fungal extract isolated from Subarnarekha River

Microorganisms (bacteria and fungi) produce several bioactive compounds those have biomedical as well as eco-monitoring activities [55].The crude extract of fungal isolates with hexane, ethyl acetate, and methanol was screened for their antimicrobial potential. The present study has evaluated the antimicrobial activity of metabolites produced by fungal endophytes against four reference human pathogenic microorganisms (*E. coli*, *Vibrio cholerae*, *Bacillus subtilis*, and *Staphylococcus aureus*).The results on the in vitro antimicrobial activities of several fungi against four different bacterial strains are given in Table 2. The results reported in this present study demonstrate the potential of fungi,

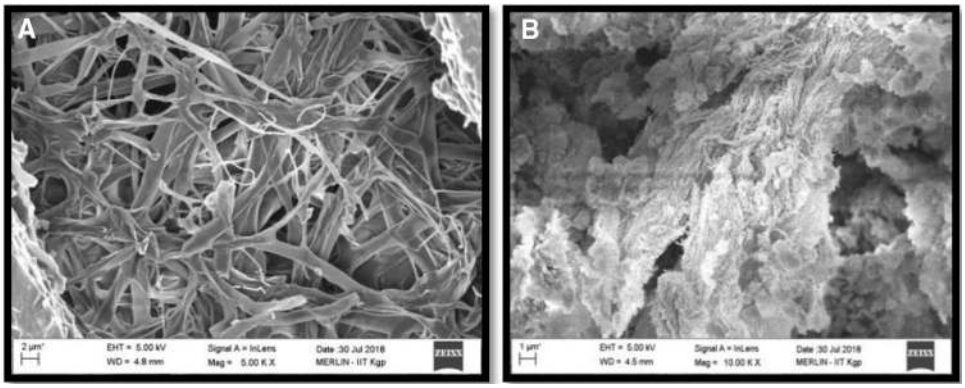

Fig. 4 SEM images of fungal strain, *Aspergillus Penicillioides* biomass (**a**) and EPS (**b**)

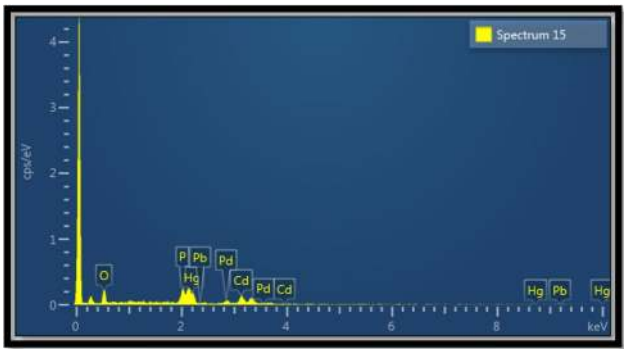

Fig. 5 Obtained EDEX spectra of F12 strain of biomass

Fig. 6 Antimicrobial sensitivity test of *Aspergillus Penicillioides* (F12) extract

Table 2 Antimicrobial activity of fungus (F12) against both gram-negative and gram-positive bacteria

Fungal name	*E. coli*	*Staphylococcus aureus*	*Bacillus subtilis*	*Vibrio cholerae*
Aspergillus penicillioides (F12)	++	++	++	++
Fusarium sp.	+	+	+	+
Penicillium sp.	+	+	+	+
Rhizopus sp.	+	+	+	+
Pythium sp.	++	+	++	++

Aspergillus sp. F12 (MN210327), showed antibacterial activity followed by *Pythium* sp. *Fusarium* sp. *Rhizopus* sp., and *Penicillium* sp. Test with the crude extract produced by the aquatic isolate showed promising results for growth inhibition of human pathogenic bacteria (Fig. 6). Therefore, it indicates that these fungi can be an important source of bioactive substances of biotechnological interest.

4 Conclusion

In the present study with the fungal isolate, *Aspergillus penicilloides* F12 (MN210327) was observed to display dual roles of antibacterial as well as heavy metal scavenging activity but most of the metal resistance fungi cannot show such antibacterial activity. Considering different research information generated out of the present study, it can be inferred that the water of Subarnarekha river in India at different locations have been polluted by the considerable amount of organic and inorganic wastes which have necessitated to undertake remedial measure for the cause of human society. The elemental concentrations of Pb, Cd, and Hg were found to exceed the permissible limits of WHO, whereas the prevailing higher abundance of bacteriological indicators in the river water was because of the discharge of wastes out of anthropogenic activities. Based on the results of water quality parameters as well as of bacterial counts, it is recommended that the water of this river in the existing state should not be used for the purpose of human use, especially as drinking water without proper treatment.

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Authors' contributions Kishalay Paria designed and performed the research experiments. Kishalay Paria and Susanta Kumar Chakraborty wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Coflict of interest The authors declare that they have no competing interests.

Availability of data and materials All data generated or analyzed during this study are included in this published article.

Consent for publication All of the authors have read and approved to submit it to SN applied science.

Ethical approval Not applicable.

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