Int.J.Curr.Microbiol.App.Sci (2022) *11*(08): 108-129

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 11 Number 08 (2022)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2022.1108.012

Green Biocide from *Boerhaavia diffusa* Leaf Extract Inhibits Biocorrosion of Mild Steel (MS1010) in Cooling Towers: Bactericidal and Electrochemical Studies

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Keywords

Mild Steel, Microbial Induced Corrosion, Cooling water Towers, Green Biocide, *Boerhaavia diffusa*

Article Info

Received:
07 July 2022
Accepted:
05 August 2022
Available Online:
10 August 2022

A B S T R A C T

In this study, we have investigated the antimicrobial effects of bacterial strains isolated and characterized from cooling water towers and biofilms niches of green biocide obtained from the plant *Boerhaavia diffusa* plant extract. Initially we have enumerated the viable bacterial cells from cooling water and biofilms of mild steel (MS1010). Various strains of heterotrophic bacteria that are isolated from viable bacterial colonies include iron oxidizing bacteria (IOB), Manganese oxidizing bacteria (MnOB), acid producing Bacteria (APB). Morphology of the isolated bacterial cells showed circular cells, slight yellow and gram positive rod cells. Specifically we have identified the selective bacterial cells as SKR-4 and SKR-7. Furthermore we have confirmed that the isolated strains SKR-4 and SKR-7 were capable of inducing corrosion of mild steel (MS1010), and it was confirmed using FITR and electrochemical studies like weight loss, polarization studies. Later we have tested the antibacterial effect of green biocide obtained from *Boerhaavia diffusa* and it was observed that 50 ppm green biocide was optimum for antibacterial effect of *Boerhaavia diffusa.* Also the same dose 50 ppm was capable of inhibiting the formation of biofilm and extracellular polymeric substances (EPS). Taken together, our results showed that *Boerhaavia diffusa* plant leaf extract has the ability to inhibit the corrosion of mild steel (MS1010) and it is due to its antimicrobial efficacy and ability to inhibit the formation of EPS. However, our results are preliminary and further studies may be required to understand its mechanism of antibacterial action and to improve its efficacy on large scale levels.

Introduction

Microbiologically induced corrosion (MIC) or bio corrosion is a phenomenon in technical systems like industries, oil fields and marine environments, thereby affects functioning of processing industries (Lavanya, 2021). Though natural corrosion process also contributes to dysfunctionality of processing industries or industrial circuits, MIC or bio corrosion contributes to 20% failures of all industrial circuit dysfunctions (Li *et al.*, 2018; Coetser and Cloete, 2005), and it accounts for 2.5 trillion USD globally for such damage (Amendola and Acharjee, 2022). MIC is distinct as it is localised in specific compartments of industrial machines and it is different from uniform corrosion. Moreover,

corrosion is a pervasive problem in natural and artificial environments like cooling water systems, causing electrochemical change i.e., conversion of normal metallic state into ionized species (Beech and Gaylarde, 1999) and also influences biocorrosion in metal alloys, ceramics and polymer composites (Lavanya, 2021 and Omar *et al.,* 2021). Indeed such electrochemical changes in metallic surfaces of industrial circuits are induced by the array of microbial cells (chemoautotrophs and/or heterotrophs) and extracellular polymeric substances (EPS). EPS assists in anchorage of microbial cells and other inorganic precipitates (derived from endogenous metal substratum) to the metallic surfaces of industrial circuits, like cooling towers (Elhousni *et al.,* 2017; MacDonald and Brozel, 2000). Microbial metabolites like sulphides, organic acids, inorganic acids, ammonia and presence of bacterial cells reducing sulfides, acid producing bacteria, ferrotrophic bacterial cells (oxidizing and reducing) and fungi collectively induces biocorrosion in industrial settings. These metabolites and microbial cells alter factors like salinity, acidity and aerobic status near the metal surfaces in systems like cooling towers and eventually cause biocorrosion (Qian *et al.,* 2019).

Apart from the above, extracellular polymeric substances (EPS) favours binding of microbial cells onto iron/metallic surfaces in industrial environments like cooling towers and thereby mediates generation of microbial bio films. MIC or biocorrosion usually occurs underneath the biofilms, and such induction of biocorrosion underneath biofilm is considered as the initial step in the induction of biocorrosion (Guo *et al.,* 2018). Components of EPS are reported to be polymers containing array of proteins, polysaccharides and lipids with functional groups to bind onto metallic surfaces and it also renders binding surfaces for iron ions on metallic surfaces, causing bio corrosion (Jin *et al.,* 2014). Notably, mild steel (MS1010) was more commonly used in industrial setting like cooling towers due to its better insulating ability, simple to use and affordable cost (Prabakaran *et al.,* 2014). However, on the other side reports denotes MS1010 is more susceptible to bio corrosion than other metals like copper in processing industrial settings like cooling towers (Li *et al.,* 2018). Hence, it is obvious that impact of MS1010 bio corrosion can significantly contribute to global economic burden due to MIC. With increasing impact of MIC on global economy (Kip and Van Veen, 2015), in recent decades various studies have been conducted globally to mitigate the MIC in processing industries (Amendola and Acharjee, 2022; Dou *et al.,* 2021). Researchers have used various natural resources (natural and modified forms) containing bactericidal efficacy to inhibit MIC in industrial setting like cooling towers and oil fields or marine environments (Wang *et al.,* 2022; Kijkla *et al.,* 2021 and Narenkumar *et al.,* 2017). In this context, we have attempted to evaluate the bactericidal and electrochemical effects of green biocide obtained from *Boerhaavia diffusa* leaf extracts in bacterial cells obtained from cooling towers of industries in vellore, Tamil Nadu, India. *Boerhaavia diffusa* (Spreading Hogweed) is a flowering plant of Nyctaginaceae family used commonly as green vegetable in India.

Materials and Methods

Sample Collection

The cooling water and bio film samples were collected from the cooling tower plant located in Ranitech Common Effluent Treatment Plant (CETP), V.C Mottur, Walajapet, Tamil Nadu. Biofilm samples were scrapped from inner cooling water tube by using a sterile surgical knife and stored carefully. About 25 liters of cooling water sample was also collected from CETP. The collected samples were transported in an ice box to EMMR laboratory at Thiruvalluvar University, Vellore. Both biofilm and water samples were then used for physicochemical and microbial characterization studies.

Enumeration of cooling water corrosive bacteria

The cooling water and biofilm samples were serially diluted (tenfold) and subjected to pour plate technique for aerobic bacterial isolation. Nutrient

agar for heterotrophic bacteria (HB), Iron agar for iron oxidizing bacteria (IOB), Mn agar for manganese oxidizing bacteria (MOB), thiobacillus agar for acid producer bacteria (AP) were used for respective bacterial cells enumeration. Cooling water and biofilm samples were inoculated and incubated at 37 °C for 24 hours (hrs). After incubation, the total viable bacterial counts were enumerated, and the bacterial population was expressed as colony forming units per ml (CFU/ml). Enumeration of bacterial cells from samples was performed using standard procedures with necessary modifications (Rajasekar Aruliah, 2014).

Biochemical Characterization of the isolates

Morphologically dissimilar dominating isolated colonies were selected randomly, to obtain pure cultures using nutrient agar. The pure cultures were maintained in nutrient agar slants at 4°C to keep the microbial strain viable. Aerobic bacterial cells isolated from medium were differentiated as per Bergey‘s Manual of Determinative Bacteriology (Cowan, 1948). To catogerise the genus of bacterial cells obtained from the purified cultures, following studies were conducted: (1) Gram staining, (2) Catalase test, (3) oxidase test, (4) pigment production, (5) nitrate reduction test, (6) indole production, (7) methyl red test, (8) Voges–Proskauer test, (9) citrate utilization test, (10) McConkey test, 21 (11) Urease activity, (12) starch hydrolysis test, and (13) carbohydrate fermentation test (Flemming *et al.,* 2016). In addition, citrate agar was used to detect the iron-reducing bacteria. Biochemical characterization of the isolates was carried out employing Himedia biochemical test kit (Mumbai) according to the manufacturer‘s instructions, to differentiate the gram positive and gram negative bacterial strains obtained from the cooling water tower mild steel (MS1010).

Extraction of Green biocide from *Boerhaavia diffusa*

Extraction of green biocide from *Boerhaavia diffusa* leaves were performed as follows: Clean, mature and healthy leaves from *Boerhaavia diffusa* plant was collected, washed finely using clean water (thrice) and again washed in sterile deionized water and shade dried (with natural air flow and mean temperature 25°C for 36h) (Vaithiyanathan *et al.*, 2018). After shade drying, the leaves were grinded using sterile pestle and mortar to obtain a fine powder and stored carefully. About 40 grams of the fine powdered plant material was added to methanol 400 ml and incubated for upto 2 days. Then the methanol was evaporated under reduced pressure and the residual contents were partitioned using chloroform ($CHCL_3$) and water. Finally, using 3% HCL the $CHCL_3$ was evaporated and then a green viscous residue, *Boerhaavia diffusa* green biocide was obtained.

Antibacterial Activity of *Boerhaavia diffusa* by agar well cut diffusion method

To evaluate the antibacterial efficacy of *Boerhaavia diffusa* green biocide cooling water bacterial strains *Bacillus firmus*, *Pseudomonas stutzeri*, *Bacillus megaterium* were cultured on a nutrient broth (Hi-media, Mumbai). Meanwhile, different concentrations of green biocide were prepared (500, 250, 125, 50, 25, 5µg/ml) for evaluating its anti-bacterial efficacy. Log phase bacterial strains from nutrient broth were streaked onto nutrient agar plates, and about 3 mm well was created on the agar plates using gel cutter. 100 µl of tested sample was added to the agar plate well and incubated 37^{o}C for 24h. Ampicillin was used as a (Positive control) and Milli-Q water (negative control) was also used. Following incubation, the bacterial inhibition zone in each plates with different bacterial strains was measured by antimicrobial scale (Hi-media). Experiment was performed using standard procedures with required modifications (Gonelimali *et al.,* 2018).

MIC and MBC determination of *Boerhaavia diffusa*

In our study, MIC was determined using standard procedure given by Clinical and Laboratory Standards Institute (CLSI), USA, (broth microtiter dilution method) (CLSI, 2018 28th edition).

Boerhaavia diffusa green biocide compound (various doses, 500 to 0.5 µg/ml) was freshly prepared in Milli-Q water, similarly ampicillin (Positive control) and Milli-Q water (Negative control) was also prepared in Luria Bertani (LB) broth in 96-well microtiter plates. Cooling water bacterial cells (SKR-4 *Pseudomonas stutzeri*, SKR-7 *Bacillus megaterium*) 5×10^5 CFU/ml was prepared in LB broth and added to 96 well microtitre plates containing green biocide and control drugs.

The plates were then incubated for 18 h at 37°C. To study MBC, 10 µl of cooling water bacterial cells from each treatment group wells with visible inhibition were diluted in LB broth and inoculated in LB agar plates followed by incubation for 24 h at 37°C to check for surviving cultures.

Biofilm Inhibition Assay

Inhibition of bio film formation by *Boerhaavia diffusa* green biocide compound was studied spectrophotometrically using crystal violet assay (Narenkumar *et al.*, 2018). Cooling water bacterial cells were maintained in Luria broth (LB) medium.

Optical density (OD) of cell culture was adjusted to $1x10^6$ CFU/ml in LB medium, and 100 µl of each sample was added to 96-well plates containing different concentrations of green biocide ranging from 500 to 5µg/ml (obtained by 2-fold serial dilution) in LB and incubated at 37°C for 24 h without shaking.

Similarly positive and negative control groups were also treated in similar fashion. Following incubation, the wells were washed with sterile PBS (Phosphate buffered saline) to ensure the removal of planktonic bacteria, and 200 µl of 0.1% crystal violet solution was added to all wells and incubated for 5 minutes.

After incubation, excess crystal violet was removed, and the plates were washed twice (using sterile PBS) and air dried. Finally 100 µl of ethanol was added to all wells to solubilize the stain, and the absorbance was measured at 595 nm using spectrophotometer (Thermoscientific India, Pvt, Ltd.).

The percentage of biofilm inhibition was calculated as follows:

Percentage biofilm inhibition = Control absorbance − Sample absorbance/Control absorbance x 100

Estimation of extracellular polymeric substances

10 ml of LB broth was prepared and inoculated with bacterial strains *Bacillus firmus, Pseudomonas stutzeri, Bacillus megaterium* obtained from cooling water. Innoculated broth were incubated at 37°C for 24 h in the following pattern.

Broth containing *Boerhaavia diffusa* green biocide treated group, positive and negative control group, control or untreated group and positive, negative control groups respectively.

Following incubation, bacterial strains from all groups were centrifuged at 5,000 rpm for 45 min to obtain the extracellular polymeric substances (EPS) from supernatant solution. To the supernatent, equal volume of Isopropanol was added and kept at 8°C for 12 h and then centrifuged at 5,000 rpm for 45 min to obtain the pellet and the pellet was suspended in 0.1 M PBS (pH 7.2). This method was performed following standard procedures as reported earlier with slight modifications (Eboigbodin and Biggs, 2008). Finally the total protein concentration in the suspension was estimated by Bradford assay (Bio-Rad, Hercules, CA) using BSA (bovine serum albumin) as standard (Bradford, 1976).

Cooling tower Mild steel (MS1010) Corrosion studies

Metal Sample Preparation

The elemental composition of mild steel (MS1010) is as follows (%): 0.37% silicon, 1.24% manganese, 0.026% sulphur 0.027% phosphorus,, 0.19% copper, 0.16% carbon 0.007% nitrogen, 0.02% aluminum, and 97.96% iron. These MS1010 coupons were exposed to the cooling tower water to examine susceptibility of the metal to biofilm formation.

Weight loss measurement

Weight loss measurement studies was performed to find the extent of corrosion rates. MS1010 coupons of 2.5 x 2.5 cm size with a hole on the top were used for weight loss experiments. The coupons were machine polished to mirror finish, degreased with trichloroethylene and rinsed with deionised water, coupons pre–weighted were immersed in cooling tower water and 1% of LB broth inoculated with enrichment of *Bacillus firmus*. Medium without bacterial strain served as control.

Similar experiment was conducted with/without *Boerhaavia diffusa* green biocide. After 10 days, the coupons were taken out and the corrosion product was scratched carefully, then coupons were pickled with Clark solution, (2%) antimony trioxide and 5% stannous chlorides dissolved in concentrated HCL at room temperature (RT) with constant stirring about 5 to 10 min, washed in deionised water and dried using air drier.

Duplicate experiments were made for each system. Final weight of the three coupons in each system was taken, and the average corrosion rates were calculated as recommended by the national association of corrosion engineers (NACE), Houston. The standard deviation (S.D) for each system was presented.

The weight loss of the metal obtained in milligrams was converted to corrosion rate in millimetre per year (mm/y) using the formula.

$$\text{Corrosion rate (mm/year)} = \frac{\text{WL (mg)}}{\text{K} \times \text{A (cm}^{2)} \times \text{T (hrs)} \times \text{D}}$$

Where

K-constant = 8.76×104
WL-Weight loss
A-Area = 2.5× 2.5
T-time in (hours)
D-density = 8.75
Mm/y-millimeter/year

Electrochemical studies polarization measurements

The MS1010 coupons of 1 x 1 cm^2 dimension with an extended stem of 15-cm length were used for polarization and impedance studies. Specimens were polished to mirror finish using emery paper 1/0 down to 4/5. The specimens were also finally degreased with trichloroethylene, followed by deionized water. Three specimens were immersed in separate 400 ml conical flasks that contained cooling water. Cooling water isolated bacterial strains Bacillus megaterium were inoculated in cooling water with 1% of nutrient medium, which was used as the experimental system, while the uninoculated specimen was used as the control system. Similar experiment was conducted by with/without *Boerhaavia diffusa* green biocide compound. Impedance measurements were carried out by Auto lab with Nova 2.1 software. MS1010 coupon of size 1 cm^2 as working electrode a Saturated Calomel Electrode (SCE) as a reference electrode and a large platinum electrode were employed for Impedance study. After a steady state was attained, an AC signal of 10 mV amplitude was applied, and the impedance values were measured for frequencies ranging from 0.1 Hz to 100 kHz. The values of Rt were obtained from the Nyquist plot. Impedance measurements were also taken on the 10^{th}day of the immersion period.

The electrodes of the same specification that employed for the impedance studies were also used for the polarization studies. Polarization measurements were carried out potentio dynamically employing an Auto lab with Nova 2.1 software. The system was allowed to attain a steady potential value for 10 min. The steady state polarization was carried out from OCP to +200 mV SCE and -200 mV SCE from the OCP using separate electrodes at a scan rate of 1800 mV/h. The polarization study was done on the 10^{th} day of the immersion period. This method was performed using standard methods reported earlier with required modifications (Narenkumar *et al.*, 2017).

Results and Discussion

Cooling water corrosive bacteria enumeration and identification

The cooling water sample collection site was presented in Fig.1. The enumeration of bacteria was done in cooling tower water and biofilm samples by pour plate technique. The total viable bacterial count of heterotrophic bacteria was 2.1 x 10^5 CFU/ml and 2.1 x 10^5 CFU/ml to attribute the cooling water and biofilm samples were respectively. The total viable bacterial count of iron oxidizing bacteria (IOB) was 2.1 x 10^5 CFU/ml and 2.1 x 10^5 CFU/ml to attribute the cooling water and biofilm were respectively. The total viable bacterial count of Manganese-oxidizing bacteria (MnOB) was 2.1 x 10^5 CFU/ml and 2.1 x 10^5 CFU/ml to attribute the cooling water and biofilm were respectively. The total viable bacterial count of Acid producing bacteria (APB) was 2.1 x 10^5CFU/ml and 2.1 x 10^5CFU/ml to attribute the cooling water and biofilm respectively. All the total viable bacterial counts of both samples (cooling water and biofilm) were tabulated and presented in the table 1. Further, bacterial strain of SKR-3 was detected using biochemical test and the results were shown in the table 2 and confirmed as *Bacillus sp*. Colony morphology was circular, convex, slightly yellow and gram positive rod (Fig.2). Other bacterial strain of SKR-7 and SKR 4 was identified and confirmed as *Bacillus megaterium* & *Pseudomonas stutzeri.*

Classification of *Boerhaavia diffusa*

Kingdom : Plantae

Phylum : Tracheophyta

Class : Equisetopsida C. Agardh

Order : Caryophyllales Juss.

Family : Nyctaginaceae

Genus : Boerhavia

Species : *Boerhavia diffusa* L.

Antibacterial activity

The green biocide was extracted from *Boerhaavia diffusa* by methanol (Fig.3). This green biocide was evaluated for its antibacterial efficacy against bacterial populations that induces cooling water corrosion of mild steel (MS1010). The entire bacterial growths zone of inhibition values were tabulated and presented as Table.3 and Fig.4. In SKR-4 *Pseudomonas stutzeri* and SKR-7 *Bacillus megaterium* bacterial strains will be treated with green biocide *Boerhaavia diffusa* to determine its ability to form bacterial inhibition zone of upto 12.0 mm and 14.0 mm. Absence of bacterial inhibition was observed in control system (without inhibitor compound). Further, positive control of Ampicillin was used for both bacterial strains to attribute the bacterial inhibition zone of 16.0 mm and 19.0 mm were respectively. In this study we have used Boerhaavia *diffusa* plant material as a source to extract green biocide and to test the same for its ability to mediate antibacterial properties against cooling water corrosion causing bacterial strains.

Analysis of Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in cooling water corrosion bacterial strains

Antibacterial activity of green biocide compound was studied using cooling water corrosion causing bacterial strains like SKR-4 and SKR-7 (Fig.5). The bacterial strain SKR-4 (*Pseudomonas stutzeri*), was highly susceptible with a lowest MIC of 25 ppm whereas SKR-7 (*Bacillus megaterium*) to showed susceptibility with highest MIC of 50 ppm. As observed from results shown in Table 4, the antimicrobial efficacy of green biocide was simultaneously compared with the standard antibiotics (Ampicillin).

Moreover, it was found that for, SKR-4 (*Pseudomonas stutzeri*), and for the MBC was same as MIC, whereas for SKR-7 (*Bacillus Megaterium)*, the results of MBC was observed to be 2-fold elevated when compared to MIC.

Antibiofilm activity of *Boerhaavia diffusa* green biocide compound

The antibiofilm potency of green biocide compound was also found against the two test strains (Cooling water corrosion causing bacterial strains (SKR-4 and SKR-7)), crystal violet staining method and CFU counting methods were employed to analyse the antibiofilm activity of the green biocide (Fig.6). Antibiofilm activity is expressed as BIC90, i.e., concentration inhibiting 90% of biofilm formation with BIC90 value of 50 ppm was obtained for, SKR-4 (*Pseudomonas stutzeri*), and 50 ppm for SKR-7 *Bacillus megaterium* (Table.5). Using CFU experimental analysis, it was found that BIC 90 concentration caused absolute death of microbial cells in all the two bacterial strains.

Extracellular polymeric substance (EPS) analysis

EPS substances were analyzed using green biocide compound treated with cooling water corrosion causing bacterial strains and compared without treatment of *B.diffusa* green biocide to the same bacterial strains (Fig.7 and Table.6). The protein content was gradually reduced in *B.diffusa* green biocide compound treated with both bacterial strains (SKR-4 *Pseudomonas stutzeri*, and SKR-7 *Bacillus megaterium*), when compared to control system (2.4 µg/ml). The present result was in support to the above anti-biofilm study. Therefore, the present study revealed that *B.diffusa* plant extraction of green biocide compound was found to have excellent potency of reduced the biofilm on the mild steel (MS1010).

Weight loss measurement

Corrosion rate of mild steel (MS 1010) in cooling tower water only, SKR-4 *Pseudomonas stutzeri*, bacterial strain were inoculated with separated cooling water system, plant extraction of biological compound was added to above cooling water system with/without inoculated bacterial strain were presented in Fig.8. The corrosion rate of MS 1010 in cooling water (control) was 0.5673mm/year.

In bacterial strain inoculated system II (cooling water and SKR-4 *Pseudomonas stutzeri*), the corrosion rate was 1.4307 mm/year (Table.7). The corrosion rate was 0.2887 mm/year. While adding to plant extraction of compound in cooling water system, in presence of bacterial strain showed the corrosion rate was 0.100 mm/year. The corroded metal surface was shown in the Fig.9 and treatment with green biocide *B.diffusa* system to cooling water system exhibited lower corrosion product formation on the metal surface when compared to other systems (untreated system). There were significant changes in total viable bacterial count and pH of the corrosion product (Table.8).

FT-IR analysis

FTIR spectrums of the surface film of the exposed metal to green biocide with/without bacterial culture of SKR-7 were shown in Fig. 10 & 11. In the aqueous extract of green biocide, occurrence of broad peak at 3300–3400 cm^{-1} indicated that the presence of OH group.

The peak at 1642 and 1380 cm^{-1} corresponded to the stretching vibrations of –C=O groups and amide groups were respectively (Fig.11). In SKR-7 with green biocide system, the peak at 2841, 2928, 1642 cm^{-1} indicated the presence of CH2 group and amide groups were respectively.

These organic functional groups are play vital for the adsorption on the metal surface. The green biocide leads to electron exchange, which was attributed to the formation of productive layer on the metal surface. FTIR revealed that green biocide was adsorbed on metal surface, with coordination of iron oxide with the functional group of green biocide (alkyl and amide groups) leading to the formation of a layer on the metal surface. This layer further inhibited the bacterial attachment on MS surface due to antimicrobial activity.

Electrochemical studies

Impedance spectroscopic analysis

Impedance spectroscopy data are presented in table

9 and Fig 13. In control system, Rt value was 32.00 Kohm.cm^2, whereas in bacterial system, the Rt value was 76.00 Kohm.cm^2. It indicates that corrosion was higher in high resistance systems. The nature of the curve in bacterial system also indicates that the corrosion is due to activation control. In cooling water inoculated with bacterial strain (SKR-3 *Bacillus firmus*) in presence of green biocide system, Rt value was 44.00 Kohm.cm^2, whereas in green biocide system, the Rt value was 54.00 Kohm.cm^2. In cooling water, the solution resistance was in the range between 18.00 and 20.00 Kohm.cm^2, which indicates that green biocide compound acts as reduction of corrosion and biofilm formation on the mild steel surface in cooling water.

Polarization Studies

Cooling water bacterial strain influenced corrosion on the mild steel coupons at interface between biofilm and metal surface were analyzed by polarization and electrochemical impedance spectroscopy method. All the values were tabulated and presented in the table.10 and Fig.13 SKR-3 *Bacillus firmus*, was inoculated with cooling water to obtained the open circuit potential (-0.752 V), Icorr,(19.88 µA/cm^2) and resistance polarization (1931.5 ohm.cm^2) were respectively. Whereas, cooling water system to exhibited I corr value was (13.93µA/cm^2) and reduced the resistance polarization (1636.4 ohm.cm^2). Other system of bacterial strain inoculated with cooling water and added with *B.diffusa* green biocide showed significant suppression of corrosion current (8.02 µA/cm^2), when compared to the bacterial strain was inoculated with cooling water system (i.e., untreated or control systems). Further, cooling water added with green biocide was obtained the Icorr value was (4.09 µA/cm^2). From that polarization result to conclude that green inhibitors acts as reduction of corrosion and biofilm formation on the mild steel in cooling water system.

In industries cooling water setups helps to reduce the heat generation in industrial circuits like oil refinery, steel production companies, food factories, petrochemical industries, chemical and power plants etc., (Rao *et al.,* 2012). To achieve efficient heat removal, the most important criteria is the optimized water circulation within the cooling water system. But, unexpectedly the cooling water system also supports the growth of various living species like planktons and benthonic growth within it. Presence of optimum temperatures in the range of 25 to 35º C and optimal pH near to neutral, required sunlight exposure and continuous supply of oxygen via aeration systems greatly enables the growth of living species within cooling water system (Liu *et al.*, 2009). Notably, the growth of microbes with cooling water is favored by initial exposure of bacterial cells from atmospheric air, water source i.e., water provided into cooling water system for optimal circulation to reduce heat, and such sources can be seawater, ground water, freshwater etc., Meanwhile the availability of nutrients and organic matters due to concentration by evaporation of cooling water, in addition to antiscalant substances and corrosion inhibitors (which are commonly used in cooling water setups) promotes the rapid microbial bloom or proliferation. In addition to the above, the surfaces of components like evaporative fill material, heat exchangers, water reservoir and cooling water pipelines serves as suitable place for the formation and growth of biofilms (Liu *et al.*, 2009; Rao 2012).

Mild steel is the most commonly used metal in construction industries as it is very affordable. On the other hand, it posses very low corrosion resistive ability in environments like aqueous and non-aqueous conditions, when compared to other metallic materials (El-Shamy *et al.,* 2009). So in recent days many studies were conducted globally to reduce the corrosion of mild steel in various industrial circuits using various biocides (Amin and Ibrahim *et al.,* 2011). Such corrosion inhibitors were usually organic molecules as they can react with the metal surfaces to enable corrosion inhibition (Banerjee and Malhotra, 1992). Green biocides are less toxic, available easily and friendly to the environment (Radojcic *et al.,* 2008).

Table.1 Total viable bacterial counts of cooling water and biofilm sample

S. No.	Samples	Number of viable bacterial counts (CFU/ml)			
		HB	**IOB**	**MnOB**	**APB**
1	Cooling tower water	$9.5x10^5$	$4.4x10^6$	$3.7\ x10^6$	$\mathbf{3.7x10^8}$
2	Biofilm 1	$2.2x10^6$	$1.0\ x10^5$	$4.0\ x10^6$	$\mathbf{7.0\ x10^8}$
3	**Biofilm 2**	$\mathbf{2.8x10^6}$	$\mathbf{1.0x10^8}$	$\mathbf{5.0\ x10^6}$	$\mathbf{1.0\ x10^6}$

Note: HB- heterotrophic bacteria, IOB-iron oxidizing bacteria, MnOB-Manganese-oxidizing bacteria, APB- Acid producing bacteria, CFU-Colony forming unit.

Table.2 Biochemical test for cooling water corrosive bacterial strains

S. No	Name of the test performed	SKR-4	SKR-7
1	Gram staining	+ve	**-ve**
2	Shape	Rod	**Rod**
3	Motility test	+ve	**+ve**
4	Catalase	-ve	**-ve**
5	Oxidase	+ve	**+ve**
6	Pigment production	-ve	**-ve**
7	Nitrate reduction	+ve	**+ve**
8	Indole	-ve	**-ve**
9	Methyl red	-ve	**-ve**
10	VP	-ve	**-ve**
11	Citrate	-ve	**-ve**
12	McConkey	+ve	**-ve**
13	Ammonia spots	+ve	**+ve**
14	Starch hydrolysis	+ve	**-ve**
15	**Acid production from different carbohydrates**		
i)	Sucrose	+ve	**+ve**
ii)	Arabinose	-ve	**-ve**
iii)	Lactose	+ve	**-ve**
iv)	Fructose	+ve	**+ve**
v)	Maltose	-ve	**-ve**
vi)	Galactose	+ve	**+ve**
vii)	Xylose	+ve	**-ve**
viii)	Mannitol	+ve	**+ve**
16	**Antibiotic resistance**		
i)	phenol	-ve	**-ve**
ii)	mercury	-ve	**-ve**
iii)	penicillin	-ve	**-ve**
iv)	**Gentamycin**	**-ve**	**-ve**

Table.3 Antibacterial activities of *Boerhaavia diffusa* green biocide compound and compared with negative and positive control against tested bacterial strains

S. No.	System	Bacterial inhibition zone (mm)	
		Pseudomonas stutzeri	***Bacillus megaterium***
1	Negative control	**-**	**-**
2	Green biocide	12.0	**14.0**
3	**Positive control**	**16.0**	**19.0**

Note: Negative control – Mill Q water, Positive control- Ampicillin, SKR-4 (*Pseudomonas stutzeri*), SKR-7 (*Bacillus megaterium*)

Table.4 Minimum inhibitory concentration and minimum bactericidal concentration of *Boerhaavia diffusa* green biocide compound (ppm)

S. No.	Antibacterial agents	***Pseudomonas stutzeri***		***Bacillus megaterium***	
		MIC	**MBC**	**MIC**	**MBC**
1	Green biocide compound	25	25	50	**50**
2	**Ampicillin**	**5**	**5**	**8**	**8**

Note: MIC-Minimum inhibitory concentration, MBC- minimum bactericidal concentration

Table.5 Biofilm inhibition assay of *Boerhaavia diffusa* green biocide compound

S. No.	Bacterial strains	Biofilm inhibition activity (%) of green biocide at various concentrations (ppm)			
		25	**50**	**75**	**100**
1	*Pseudomonas stutzeri*	90	100	100	**100**
2	***Bacillus megaterium***	**60**	**90**	**100**	**100**

Table.6 Estimation of extracellular polymeric substance (EPS) analysis from bacterial cultures with/without *Boerhaavia diffusa* green biocide compound

	B	**S1**	**S2**	**S3**	**S4**	**S5**	**SKR-7 (*Bacillus megaterium***			**SKR-7 +with Green biocide**		
							T1	**T2**	**T3**	**T1**	**T2**	**T3**
1.Stock standard solution	_	0.2	0.4	0.6	0.8	1.0	10	50	100	10	50	**100**
2.concentration	_	20	40	60	80	100	10	50	100	10	50	**100**
3.Distilled water	1.0	0.8	0.6	0.4	0.2	_	990	950	900	990	950	**900**
4.volume of alkaline copper	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	**4.5**
Allow to stand from 10 mins at room temperature												
5.Volume of folin"s phenol	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	**0.5**
Allow to stand from 10 mins at room temperature												
6.Optical density at 640nm	**0.000**	**0.092**	**0.164**	**0.334**	**0.473**	**0.573**	**0.560**	**0.830**	**0.999**	**0.099**	**0.121**	**0.359**

Table.7 Corrosion rate of mild steel (MS 1010) in different systems

S. No.	System	Immersion periods(day)	Average weight loss(mg)	Corrosion rate (mm/year)	Form of corrosion
1	400ml of cooling tower water immersion on MS 1010 coupon (2.5 x 2.5 cm)	20	17	0.5673	**Uniform corrosion**
2	400ml of cooling tower water immersion on MS 1010 coupon and inoculated with bacterial strain (SKR-3 *Bacillus firmus*)	20	30	1.4307	**Pitting corrosion**
3	400ml of cooling tower water immersion on MS 1010 coupon and inoculated with bacterial strain (SKR-3 *Bacillus firmus*) in presence of green biocide (*Boerhaavia diffusa*)	20	21	0.2887	**Slime corrosion**
4	**400ml of cooling tower water immersion on MS 1010 coupon and with presence of green biocide (*Boerhaavia diffusa*)**	**20**	**40**	**0.100**	**No corrosion**

Table.8 Total viable bacterial count and pH from corrosion product on mild steel in cooling water system

S.No	Systems	Corroded Mild steel surface (pH)	Water medium (pH)	Total viable bacterial counts (CFU/cm2)
1	400ml of coolingtower water immersion on MS 1010 coupon (2.5 x 2.5 cm)	10	7.0	**6.2×10^5**
2	400ml of coolingtower water immersion on MS 1010 coupon and inoculated with bacterial strain (SKR-3*Bacillus firmus*)	9.0	9.0	**7.0×10^5**
3	400ml of cooling tower water immersion on MS 1010 coupon and inoculated with bacterial strain (SKR-3 *Bacillus firmus*) in presence of green biocide	9.0	9.0	**7.9×10^5**
4	**400ml of cooling tower water immersion on MS 1010 coupon and with presence of green biocide (*Boerhaavia diffusa*)**	**9**	**8.0**	**4.1×10^5**

Fig.1 Photograph view of RANITECH in cooling tower industrial system and water sample collection

Table.9 Electrochemical impendence parameters for mild steel 1010 coupons

S.No	Systems	RS(Ω)	Rt(Ω)	Rct(Ω)
1	Cooling water	22	32	**10**
2	Cooling water +bacterial culture	26	76	**51**
3	Cooling water +bacterial culture+ green biocide	17	44	**27**
4	**Cooling water + green biocide**	**20**	**54**	**34**

Note: Rs solution resistance, Rt total resistance, Rct charge transfer resistance

Table.10 Potentiodynamic polarization parameters for mild steel 1010 coupons

S.No	System	Ecorr (v)	Icorr (A/cm2)	βa (mV/dec)	Bc (mV/dec)	Rp (kΩcm2)
1	Cooling water	-0.710	13.93	0.193	0.072	**1636.4**
2	Cooling water +bacterial culture	-0.752	19.88	0.286	0.127	**1931.5**
3	Cooling water + bacterial culture + green biocide	-0.709	8.02	0.065	0.103	**2168.1**
4	**Cooling water + green biocide**	**-0.745**	**4.09**	**0.142**	**0.103**	**6373.1**

Note: Ecorr corrosion potential, Icorr corrosion current, Rpresistance polarization

Fig.2 Photograph view of isolated bacterial strain of *Bacillus megaterium* and *Pseudomonas stutzeri* from cooling water sample

Bacillus Megaterium Pseudomonas stutzeri

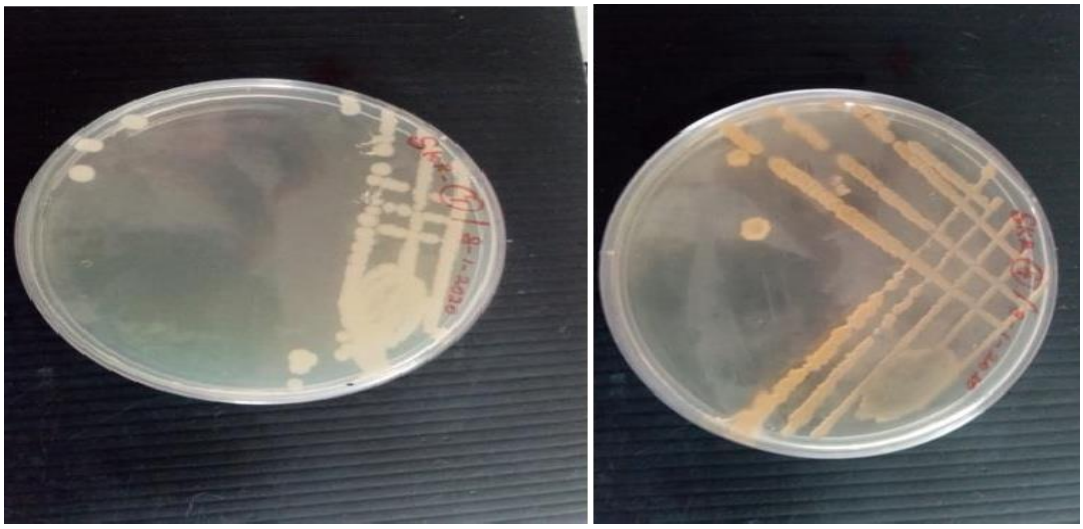


Fig.3 Photograph view of extraction of green biocide compound from *Boerhaavia diffusa*

Fig.4 Photograph view of antibacterial activities of *Boerhaavia diffusa* green biocide against tested bacterial strain by Muller Hinton agar diffusion plate.

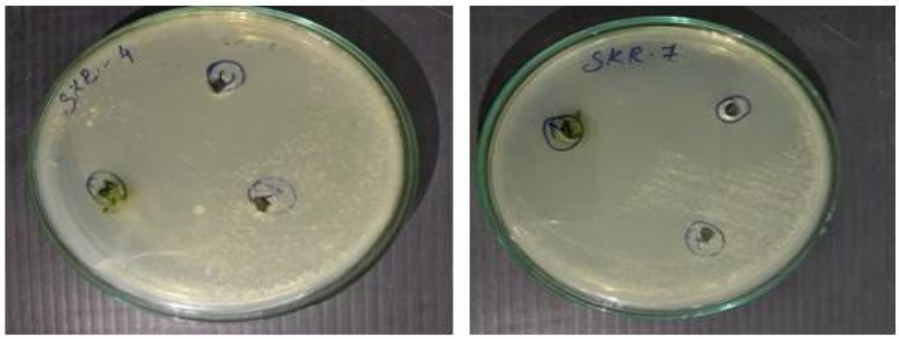


Fig.5 Visual images of Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) studies on cooling water bacterial strain with/without green biocide compound

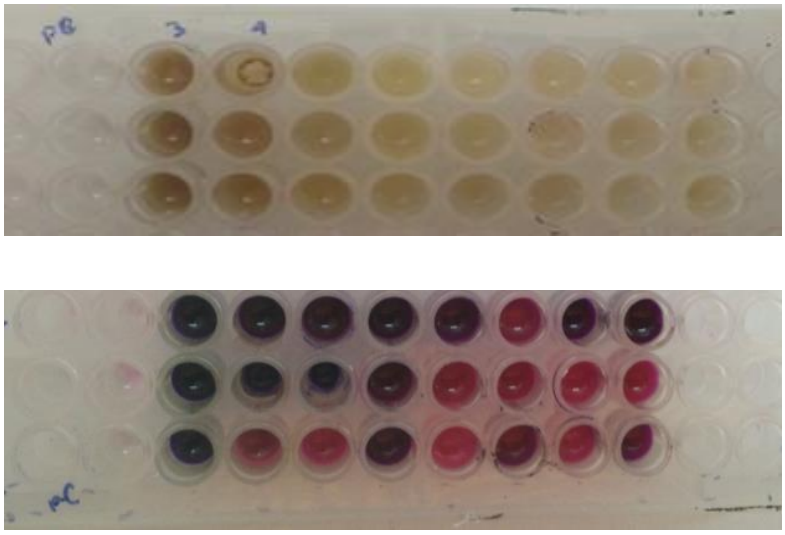


Fig.6 Visual images of biofilm inhibition assay on cooling water bacterial strain with/without *Boerhaavia diffusa* green biocide compound

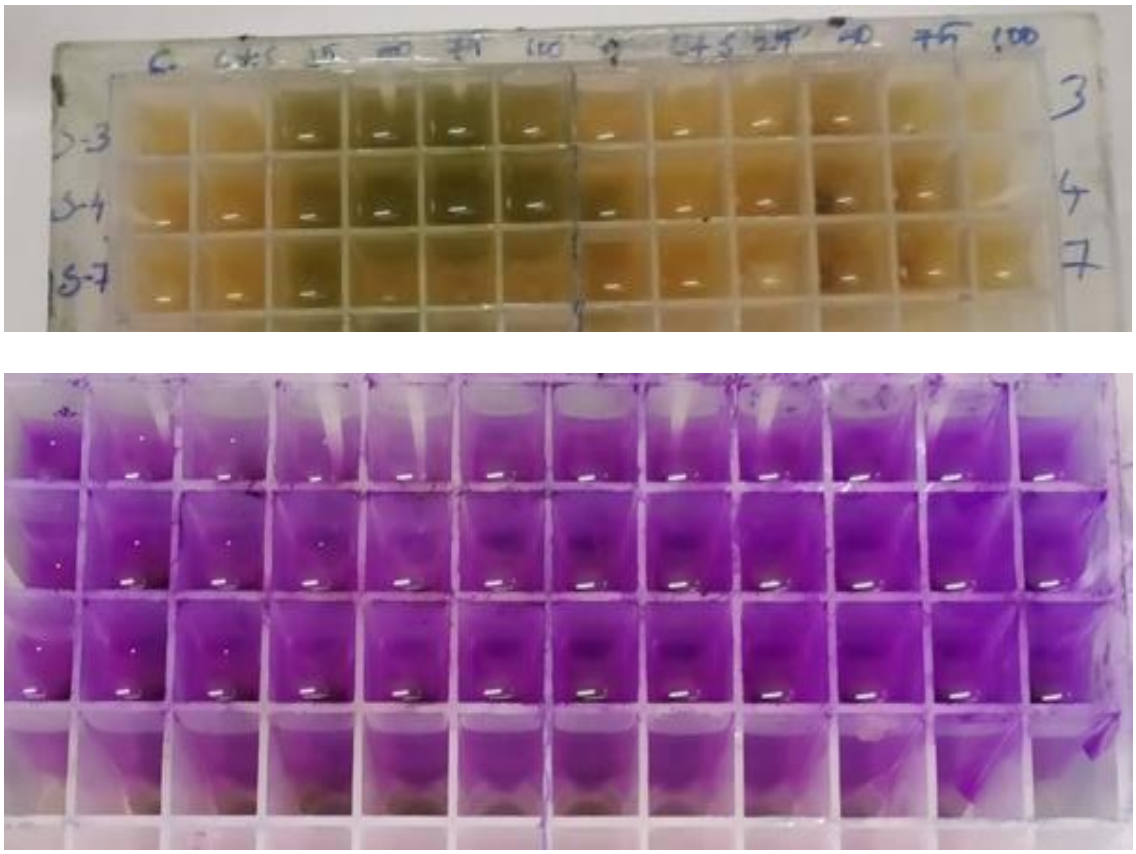


Fig.7 Visual images of estimation of extracellular polymeric substance (EPS) analysis from bacterial cultures with/without *Boerhaavia diffusa* green biocide compound.

Fig.8 Experimental setup for weight loss measurement of mild steel coupon in cooling water at various systems.

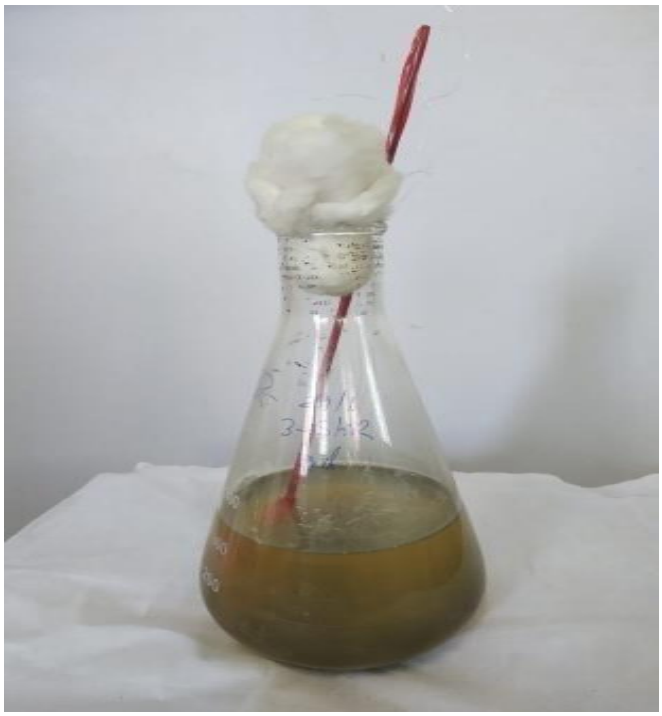


Fig.9 Photograph view of corroded mild steel surface at various weight loss experimental systems

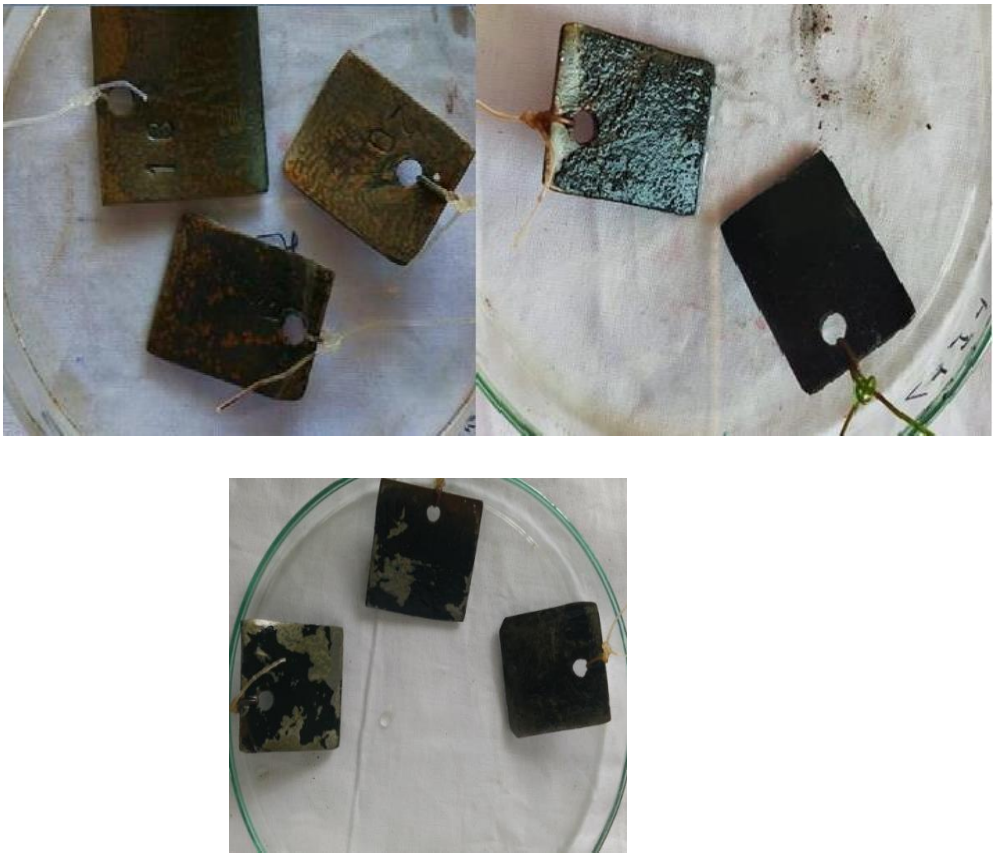


Fig.10 FTIR spectrum of green biocide compound from *Boerhaavia diffusa*

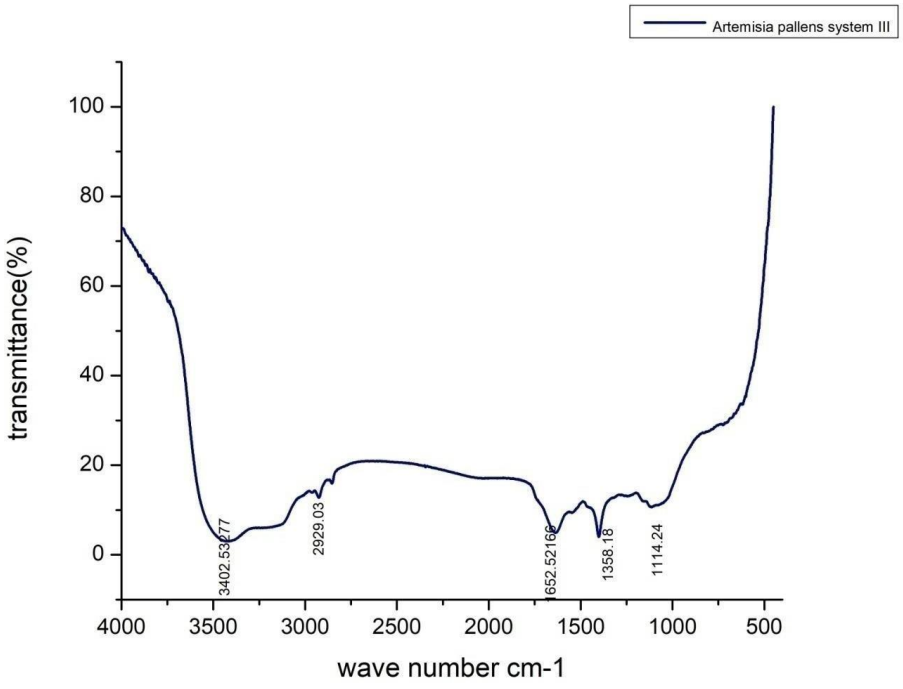


Fig.11 FTIR spectrum of surface film formed on mild steel 1010 coupons of bacterial strain with *Boerhaavia diffusa* green biocide system

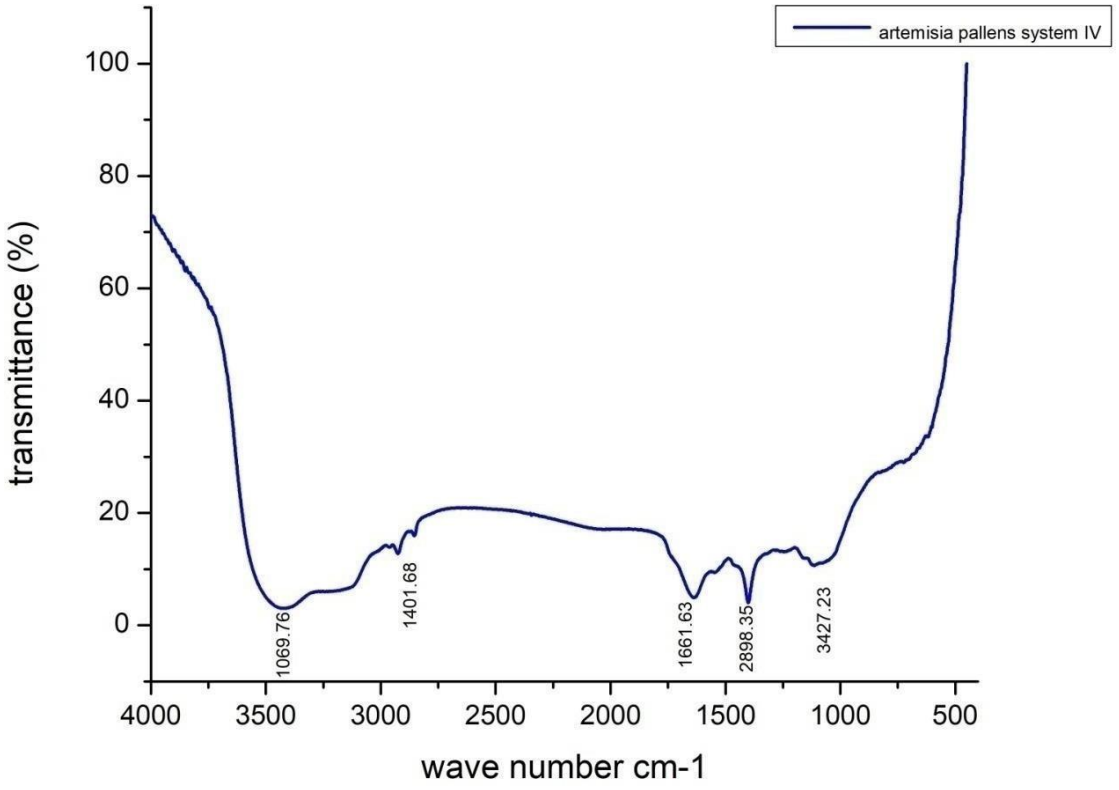


Fig.12 Nyquist plots of mild steel 1010 coupons collected from various systems

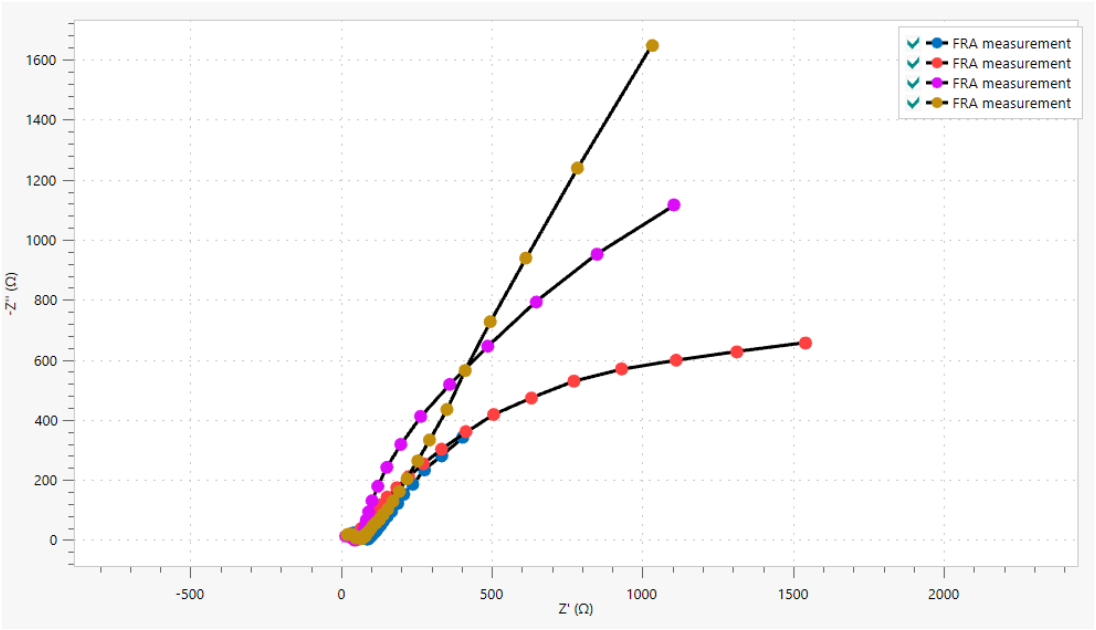
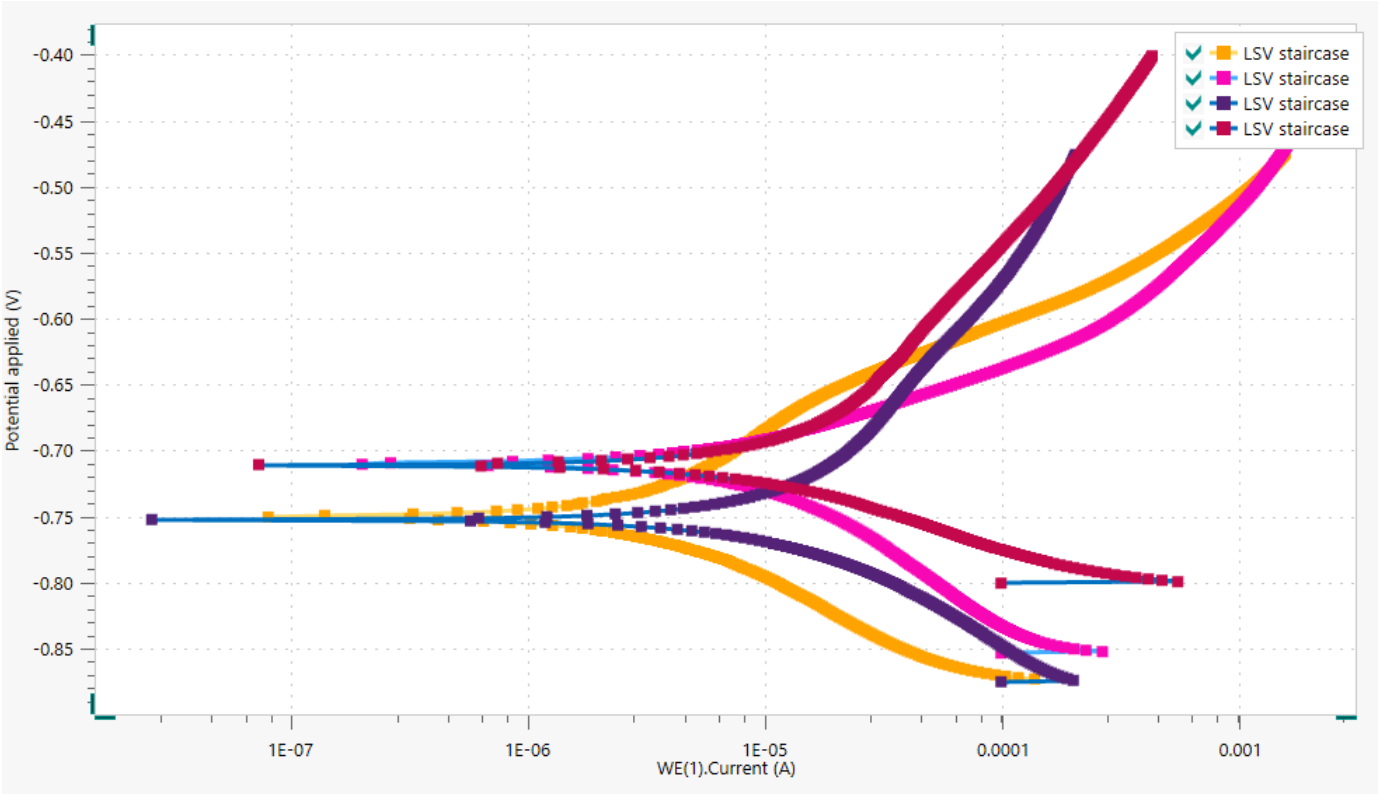


Fig.13 Tafel plots of mild steel 1010 coupons collected from various systems



Sources of plant products like clove, horse radish, tamarind, cinnamon, onion, and garlic are tested as corrosion inhibitors and also as green biocides in acidic experimental conditions. More importantly these plant derived components as biocides are found to be most compatible with treatment plants (like cooling water systems) of industrial circuits (Elhousni, *et al.*, 2017). In this context, in our present research investigation, we have employed the plant the extract of *Boerhaavia diffusa as a* green biocide to control biofilm formation and MS corrosion (MS1010).

Understanding the status of microbial population which is known as microbial monitoring is considered as important process in the analyses or studies investigating the effectiveness of antifouling or anti-MIC treatments. Biofilms are considered to be complex communities of microbial cells which are attached on the endogenously produced matrix of extracellular polymeric substances (EPS) which confers three dimension structures to the biofilms (Flemming and Wingender, 2010). Biofilms are gel like structure, containing network of polymeric chains with intra and intermolecular linkages, generated high water retaining capability. This polymeric biofilms can be heterogeneous in nature and can comprise diverse biopolymers with various chemical and physical properties. The components of biofilms were usually contains polysaccharides, proteins, amyloids, extracellular nucleic acids and amphiphilic compounds such as glycolipids and peptidolipids (Sutherland, 2016).

In the present study, our demonstrated results revealed the minimal inhibitory concentration (MIC) required for *Boerhaavia diffusa* green biocide to inhibit the growth of bacterial strains was about 40 to 50 ppm. Similar to our results, results of Kim and Park (2013) and Parthipan *et al.*, 2021 reported and stated that green biocides from ginger extract and *Syzygium aromaticum* affected the growth of bacteria and it also inihibited the biofilm formation by modulating or changing their phenotype

characterization. In this study, the aqueous extracts of ginger green biocide inhibit the bacterial growth on surface of mild Steel (MS1010). Similarly to the above, in our experiments we have found that 0 ppm of *Boerhaavia diffusa* was recognized as a dosage having significant antibacterial activity (observed as clear zone of inhibition, with 5 mm).

Similarly, formation of biofilms was determined using microtiter plate assay using cresyl violet staining experiments. It is well known that bacterial strains have the ability to form or develop biofilms in the metallic surfaces. However, as we hypothesized, the green biocides can reduce or hinder or inhibit the formation of biofilms on metallic surfaces via different biochemical mechanisms or via altering the physical factors required for their attachment to the surfaces of the mild steel (MS1010) in cooling water compartments. As expected in our experiments we have found that green biocide extract of *Boerhaavia diffusa* significantly inhibited the biofilm formation by bacterial strains. This ability of green biocides to inhibit or reduce or prevent biofilms formations on metallic surfaces is correlated to its anti-bacterial efficacy. This could be via generation of free radicals that can damage components required for of bacterial cell life or other organic substances from the green biocides that can penetrate the bacterial cell membrane and thereby interferes with the bacterial cell metabolism. Similar to our results other reports have also documented such antibacterial effects from other green biocides (Kim and Park 2013). So with the ability to inhibit bacterial cellular process, we hypothesized that our *Boerhaavia diffusa* green biocide can also inhibit the generation of extracellular polymeric substances (EPS). As we hypothesized in our studies EPS generation was greatly inhibited and this shows the bacterial metabolism is affected. In addition to the above EPS inhibition property of *Boerhaavia diffusa,* the development of biofilms was also significantly retarded by its treatment. The observed green biocide anti-biofilm property of *Boerhaavia diffusa* may be due to inhibition of regulation gene c-di-GMP levels in bacterial cells, however, we have not tested in our experimental studies (Kim and Park, 2013).

In support of the above all other results of our experiment are in support with the antimicrobial and corrosion inhibition ability of *Boerhaavia diffusa* green biocide. Results of EPS formation showed inhibition efficiency between 60 to 70%. FITR results showed the adsorbent of *Boerhaavia diffusa* green biocide onto the metallic surfaces. Furthermore studies like Weight loss, electrochemical studies, surface analysis studies, FITR analysis showed the bacterial strains which we have, isolated and characterized as heterotrophic bacteria were capable of inducing corrosion in the surface of mild steel (MS1010).

Taken together in our study, isolation and identification of corrosion causing bacterial strain from cooling water and the antibacterial efficacy of green biocide (*Boerhaavia diffusa*) and its efficiency as corrosion inhibitor inhibition of MS 1010 were investigated. From the preliminary characterization and antimicrobial studies, we have observed that the isolated bacterial strains SKR-4 and SKR-7 were capable of causing biocorrosion in mild steels (MS1010) of cooling water towers. Notably, our green biocide extract obtained from *Boerhaavia diffusa* was capable to inhibit or reduce the biocorrosion of MS1010 and this is attributed to its ability to exert antibacterial efficacy by directly interfering with bacterial cell metabolism.

It concluded the present study have identified the following bacterial strains *Bacillus megaterium* & *Pseudomonas stutzeri* using biochemical characterization studies from cooling tower water samples. Antibacterial study to confirmed that our *Boerhaavia diffusa* extract green biocide compound have excellent antibacterial activity upon cooling water bacterial strains. Moreover our, FTIR results confirmed that *Boerhaavia diffusa* extract as green biocide compound binds metal surface iron oxide co-ordination complex, forming an organic productive layer, which inhibits biofilm and exhibit controlled MIC. In this study, weight loss, electrochemical study, and surface analysis FTIR of data showed that isolated bacterial strains like SKR-

4 and SKR-7 were capable of causing corrosion of MS 1010, and these phenomena's were inhibited by *Boerhaavia diffusa* extract as green biocide compound at the optimum concentration of 50 ppm in cooling water systems.

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How to cite this article:

Lavanya, S., M. Jayanthi, D. Prakash and Kavitha Rani, M. 2022. Green Biocide from *Boerhaavia diffusa* Leaf Extract Inhibits Biocorrosion of Mild Steel (MS1010) in Cooling Towers: Bactericidal and Electrochemical Studies. *Int.J.Curr.Microbiol.App.Sci.* 11(08): 108-129.
doi: https://doi.org/10.20546/ijcmas.2022.1108.012