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Original Research Article

Consequence of *Anacardium occidentale* and *Garcinia kola* Extracts on Sulphate Reducing Bacteria and Corrosion of Mild Steel

Nwachukwu I. N[1*], Okafor J. U[1,2], Ihejirika C. E[3], Chinakwe E. C[1]

[1]Department of Microbiology
[2]Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa
[3]Department of Environmental Sciences Federal University of Technology, Owerri PMB1526 Imo State, Nigeria

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***Corresponding author:** Nwachukwu I. N
Department of Microbiology

Abstract

The effect of *Anacardium occidentale* (Cashew) leaf and *Garcinia kola* (bitter kola) seed extracts on the corrosion of mild steel in Sulfate Reducing Bacteria (SRB) environment. Enrichment of SRB was achieved by anaerobic incubation of waste water sample from gutter along old market road Owerri, Imo State Nigeria in BmA medium and BmA medium without iron. Weight loss method was used to determine corrosion. The specific growth rate and cell biomass of SRB in batch culture was determined. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts were also determined using standard methods. Results showed measurable weight loss of mild steel samples as a result of corrosion by SRB. *Garcinia kola* extract had inhibitory effect on SRB at concentrations above 50mg/ml while *Anacardium occidentale* extract showed no inhibition capacity at 200mg/ml. The MIC for the *Garcinia kola* extract was at 50mg/ml and MBC at 100mg/ml while there was no observable MIC and MBC value for *Anacardium occidentale* extract. The cell biomass of the SRB in a batch culture containing *Garcinia kola* extract was observed to decrease over time, while *Anacardium occidentale* extract showed an increase in cell biomass with no decrease over time. *Garcinia kola* extract also reduced the specific growth rate of SRB significantly while *Anacardium occidentale* extract did not show any significant reduction in the specific growth rate of the SRB cultures. *Garcinia kola* may have potentials for use in the development of benign natural products for mitigation of microbial induced corrosion of mild steel.

Keywords: Corrosion, Sulphate Reducing Bacteria, *Anacardium occidentale* and *Garcinia kola*.

1.0 INTRODUCTION

Sulfate reducing bacteria (SRB) are prokaryotic obligate anaerobes that use sulphur, sulphate or other oxidized sulfur compounds as oxidizing agents when decomposing organic material [1]. They grow in environments that are rich in sulfate (SO_4^{2-}) and have the metabolic machinery that reduce it gradually to sulfide(S^{-2}) passing through intermediate steps of incomplete sulfur oxidation in which sulfides are formed [2]. They play major role in contamination of petroleum products and anaerobic corrosion of steel by the production of sulfide which is highly reactive and toxic [3]. Anaerobic corrosion of metal is a major economic problem and is associated with the activity of SRB [4]. This activity can lead to perforation and leakage of stored products held in metal reservoir. SRB have also been implicated in corrosion of machine parts used in various manufacturing and processing plants [5]. Microbial activity leads to fuel degradation which may in turn result to problem of unacceptable level of turbidity, corrosion of storage tanks, pipelines and souring of stored products [6].

Sulphate reducing bacteria enhance metal corrosion by reducing sulphate and sulphur containing compounds to hydrogen sulphide which in turn reacts with water to produce an acid condition, thereby accelerating corrosion process [3]. This has led to the increasing interest in bio-corrosion and means of mitigating the effects in industrial environments. Microbiologically influenced corrosion has been estimated to account for 20-30% of all internal pipeline corrosion costs [7]. Metals corrosion has become a constant and persistent problem, often difficult to eliminate completely [8]. The use of inhibitors has been adopted in the industries for a long time now in quest for excellent anti-corrosive agents [9]. A corrosion inhibitor is a substance which when introduced in a small

concentration to an environment, decreases the corrosion rate of metal exposed to that environment [10]. Currently, research activities are focused on finding a replacement for inorganic [11] and organic metal corrosion inhibitors. The addition of corrosion inhibitors effectively shields the metal against an acid invasion [12]. Most inhibitors are not environmental-friendly but rather toxic and expensive. Therefore, the new approach on the use of non-toxic or environmentally safe corrosion inhibitors such as extracts is essential to overcome this problem. Chemical inhibitors of corrosion have been used over years, however interest in the use of extracts from plants in curbing corrosion is recent. It is believed that certain plant extracts may possess active inhibiting chemical compounds. Plant extracts are one of the sources targeted for the replacement of inorganic and organic inhibitors. Some of these plant extracts are *Carica papaya* and *Camellia sinensis* leaves [13, 14], *Cola acuminata* and Camellia [15], *Saccharum officinarum* juice extract [16], Groundnut leaves extract [17], Katemfee Seed [18], and Kola extract [19].

Anacardium occidentale (Cashew) is a medium-sized (6-9m high) spreading-evergreen tree which is widely grown in the tropics for its edible fruits and nuts. The nut shells contain commercially important resinous oil. The fruit consists of a fleshy, red or yellow, pear-shaped receptacle termed the 'apple' at the distal end. *Garcinia kola* or bitter kola is commonly found in the rain forests of West Africa and belongs to the Malvaceae plant family of the cocoa family. The seeds of the fruit are called bitter kola. It grows to a height of about 12 - 14m and produces red, yellow or orange colored fruit. Each fruit contains 2 to 4 yellow seeds and a sour tasting pulp. The seeds when chewed have a bitter astringent taste. In very many cases, the corrosion inhibitive effect of some plants' extracts has been attributed to the presence of tannin in their chemical constituents [20]. Associated with the presence of tannin in plant extracts is the bitter taste in the bark and leaves of the plants. Similarly, kola nut tree's chemical composition consists of caffeine (2.0-3.5%), theobromine (1.0–2.5%), theophylline, phenolics–such as phobaphens, epicachins, D-catechin, tannic acid (tannin), sugar –cellulose and water. As reported in some previous studies [14], tannin is known to possess corrosion inhibitive properties on metals – particularly, mild steel. Phytochemical analysis of the crude extracts revealed presence of flavanoids, tannins and phenolic compounds and these components are responsible for the anticorrosion activity of the extracts. This study investigates the use of cashew leaf and bitter kola seed extracts as possible inhibitors of microbial influenced corrosion of mild steel in anaerobic environments.

2. MATERIALS AND METHODS

2.1. Sample Collection

Waste water samples which smelled hydrogen sulfide were collected in sterile universal containers from gutter along old market road Owerri, Nigeria. The water samples were capped quickly after collection to prevent air exposure. The samples were labeled and transported to the microbiology laboratory for the investigation.

2.2. Collection and Extraction of Plant Materials

Fresh leaves of *Anacardium occidentale* (cashew) were collected from a tree behind the Faculty of Engineering at Federal University of Technology Owerri (FUTO), Imo State Nigeria. The seeds of *Garcinia kola* (bitter kola) were bought at a popular market in Owerri, Imo State called Ekeonuwa market. One kilogram (1kg) of each sample was obtained. The plant samples were identified and authenticated by a botanist at the Department of Crop Science, Federal University of Technology Owerri. The fresh leaves of cashew were cut, oven dried at 50^0C and finely blended into fine powder using a blending machine. Of the ground samples, 100g were extracted by separately soaking in different 400 ml containers containing 100ml sterile water for 24 hours. These were filtered using watmann filter #2. The filtrate was left to evaporate to dryness at room temperature. They were subsequently and stored in clean air tight bottles and refrigerated for further use. A final concentration of 1200mg/ml of the extract was obtained as stock.

2.3. Media Preparation

The water samples were inoculated into B Acetate medium (BmA) for the enrichment of SRB with acetate as its carbon source [21]. The medium contained: KH_2PO_4,0.50g; NH_4Cl, 1.00g; $Cacl_2$, 1.00g; Na_2SO_4, 1.00g; $MgSO_4.7H_20$, 2.00g; $FeSO_4.7H_2O$, 0.50g; Yeast Extract, 1.00g; Sodium acetate, 3.50g; Ascorbic acid, 0.10g; Thioglycolic acid, 0.10g in 1 liter of distilled water. B Acetate medium (BmA) without iron was also used for SRB enrichment and this was prepared the same way as BmA above except that $FeSO_4.7H_2O$, 0.50g was not added. This was stirred with a magnetic stirrer and filtered using filter paper (Whatmann #1, UK). The pH of the medium was adjusted to 8.5 to determine the suitable growth condition for SRB enrichment and this was done by the addition of 1M NaOH or 1M HCl solution. A double strength B Acetate medium (BmA2X) was prepared the same way as the preparation of BmA medium, except that BmA2X contained twice as much ingredient dissolved in 1 Liter of distilled water. The prepared medium was finally autoclaved at 121ºC for 15minutes and was cooled down to room temperature.

2.4. Sample Inoculation

Tenfold serial dilutions of the wastewater samples was done and 5ml of dilution factor 10^{-3} labeled D1 was inoculated into sterile bottles containing BmA medium with $FeSO_4.7H_2O$ and incubated at 30^0C for 7 days. The growth of SRB was confirmed by observing the changes in color of the BmA medium. A confirmation test for SRB growth was done by inoculating 5ml of SRB culture from D1 into a fresh BmA medium without $FeSO_4.7H_2O$ and incubated at 30^0C for 7days. It was further sub cultured into another

fresh BmA medium at 30^0C for 7 days and observed for further color change. Positive cultures of SRB was also inoculated into a BmA medium [21, 22], using pyruvate as the only carbon source in the medium to determine if SRB uses pyruvate as carbon and energy source. The pyruvate in the later BmA medium replaces sodium acetate in the previous medium. Cultures of SRB were gram stained, viewed and identified under the microscope.

2. 5. Corrosion Experiments by Weight Loss

Mild steel coupons measuring 1inch square were obtained from engineering workshop FUTO. They were washed properly using distilled water, detergent and rinsed in 95% alcohol. They were weighed to determine their initial weight before introduction into the SRB environment. The mild steel coupons were thereafter, introduced into a BmA medium without $FeSO_4.7H_2O$ enabling the coupons to serve as source of iron for the SRB cultures. Replicate mild steel coupons were withdrawn every 7days for a total period of 35 days following inoculation with SRB. On withdrawal, the coupons were washed properly using distilled water, detergent and rinsed in95% alcohol to remove any grease on them, air dried and reweighed. Two controls were setup the first contained only mild steel while the second contained mild steel in water. All the experiments were performed at room temperature. The tests were run in triplicate.

2.6. Effect of Extracts on the Corrosion of Metals by SRB

Five equal measures of the BmA medium were put in different 250ml beakers. They were inoculated with equal volumes of previously enriched cultures of SRB. Different concentrations of each of the extracts were put in all the beakers except for one without extract which was used as control. The weighed mild steels after cleaning were separately and fully immersed for 25 days in each of the beakers containing the media. The mild steels were removed every 5 days, washed with distilled water, rinsed with methanol, dried and re-weighed. This was done for both the cashew and bitter kola extracts.

2.7. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Extracts

MIC of *Anacardium occidentale* and *Garcinia kola* extracts on SRB was determined by broth dilution method using the BmA. Equal volumes of Cultures of previously enriched for SRB were used for inoculation. The following different final concentrations (mg/ml) of freshly prepared extracts/media+inoculums Vols, 3.125mg/ml, 6.25mg/ml, 12.5mg/ml, 25.0mg/ml, 50.0mg/ml, 100.0mg/ml and 200.0mg/ml were used. Inoculum control (without extract), medium control (without inoculum) and chloramphenicol control (with antibiotics added) were also used. All bottles were labeled, sealed with paraffin and incubated for 7 days at 30ºC. After incubation, the bottles were checked for the growth of SRB. The lowest concentration of extract that prevented the blackening of the medium or formation of black precipitate of iron sulfide (inhibited growth of SRB) was taken as the MIC value. The MBC was determined by transferring 5ml of SRB culture from the MIC bottles into universal bottles (30ml) containing different final concentration of extracts. BmA medium was used to fill up the bottles, capped and sealed with paraffin to provide anaerobic condition and incubated for 7days at 30ºC. The bottles were also checked for the growth of SRB. The lowest concentration of extract that prevented blackening of the medium or formation of black precipitate of Iron sulfide (killed the SRB) was taken as the MBC value.

2.8. Determination of Specific Growth Rate of SRB Using the Breed's Method

The specific growth rate of SRB in batch culture was determined by inoculating 5ml of SRB culture into four universal bottles (30mls) containing BmA medium without iron on day 1. The first bottle labeled as bottle A was used as control with no extract added. Ten (10mls) of SRB culture was pipetted from the second bottle labeled as bottle B. The removed broth was replaced with 5ml of 100mg/ml concentration of extract and 5ml of BmA2X medium. On day 4,10ml of SRB culture was also pipetted from the third bottle labeled as bottle C and 5ml of extract followed by 5ml of BmA2X medium was added. On day 7, 10ml of culture was also pipetted from the fourth bottle labeled as D and 5ml of extract followed by 5ml of BmA2X medium was injected into the bottle. The bottles were incubated at 30ºC for 7days. Sampling was done every 3days beginning from the first day of experiment until day 7 to determine the number of SRB cells by using breed's methods [23]. Specific growth rate (µ) of SRB was determined by the formula:

$$\mu = \frac{\log x_t - \log x_o}{t \times 24}$$

Where $\log x_t$ = log number at time $t = t_1$
$\log x_o$= log number at time t = 0
T = time (hour)

Breed's method [23], was done by discharging 10µl from each bottle using sterile syringe and spread on a glass slide. The smear was heat –fixed and was flooded with safranin for 1minute. The slides were washed with distilled water and air dried and was observed under light microscope at 1000X magnification. The number of cells was counted by observing 4 to 5 of field under the microscope. Dilution was necessary if the number of cells per field was too large. The number of cells/ml (cell biomass) of original suspension was determined according to the following formula.

Number of cell/ml =

$$\frac{3.781 \times 10^5 \times 1 \times y}{10^{-z}}$$

Where y is the average number of cell count
10^{-z} is the Dilution factor.

3. RESULTS AND DISCUSSION

3.1. SRB Enrichment

BmA medium changed from clear pale yellow to black after 5days of incubation of the waste water samples which indicates the growth of SRB. The confirmation test for SRB in BmA medium without $FeSO_4.7H_2O$ showed a very slight color change from pale yellow to turbid yellow since the media didn't contain $FeSO_4.7H_2O$ to react with sulphide. SRB was observed to grow using pyruvate as its carbon source which was indicated by the change in color from pale brown to black. The Gram staining showed that they are Gram negative and rods/rodcocci in shape. The unpleasant odor of the culture is another indication of SRB growth [23]. H_2S has a pungent smell and it is produced by SRB.

3.2. Effect of Extracts on Weight Loss and Corrosion by SRB

There was an observable blackening of the mild steels and medium after the specified period of incubation which led to microbial induced corrosion as indicated by the weight loss of the mild steel as shown in Figure 1.0. The results of corrosion rate of mild steels with time for the coupons inoculated into SRB culture containing various concentrations of *Anacardium occidentale* extract showed there was only a little insignificant inhibition of corrosion. This is shown in Figure 2.0. The concentration of the extracts did not affect the corrosion rate significantly. The positive control (without extract) clearly gave a higher corrosion rate. These results indicate that cashew may have an inhibiting property. However, it is uncertain if the optimum concentration needed for effective corrosion inhibition have been reached with the concentrations of extract tested. Therefore, the low concentration of the extract could not ensure adequate inhibitive barrier for corrosion protection.

Garcinia kola extract at a concentration above 50mg/ml had a better corrosion inhibition while at a concentration below 25mg/dl showed the least corrosion inhibition effect to the test specimens inoculated into SRB cultures as shown in Figure 3.0. The corrosion rate values decreased with increase in the exposure time. This could be as a result of the devitalizing of the test medium by the corrosion products which might have contaminated the medium and hence stifled the corrosion reactions. From the graph, the initial corrosion rate value for the highest concentration of extract (200mg/dl) on the fifty day was at 9mm/yr, which on the last day of experiment had reduced to 3.0mm/yr. The *Garcinia kola* extract could be considered to be effectively inhibitive relatively for the test specimen used in this study. The weight loss result in this study is different from a study where the weight loss showed that SRB inhibits the corrosion of the steel [24].

3.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC for the *Garcinia kola* extract was a 50mg/ml and the MBC were100mg/ml. Control B_2 (without extract) resulted in rapid SRB growth which was expected. The *Anacardium occidentale* extract did not inhibit the growth of SRB at any of the concentrations tested, thus there was no observable MIC and MBC value. Medium control (B_3) showed no growth indicating that the medium was stable throughout the incubation period. The control B_1 (with chloramphenicol) showed no growth, and indicates that the SRB growth was inhibited by the antibiotic. These are as shown on Tables 1, 2 and 3

3.4. Cell Biomass and Specific Growth Rate of SRB

Bottle A which is the control showed the highest increase in cell biomass as expected because no extract was added as shown in figure 4.0. The cell biomass of the SRB in the batch culture with *Garcinia kola* extract was observed to reduce over time for bottle B and C, while bottle D showed an initial increase in the cell biomass but decreased over time. The cell biomass of SRB for culture containing *Anacardium occidentale* extract had an observable increase in cell biomass and subsequently remained fairly constant over time. There was no significant variation in cell biomass for all the bottles (A, B, C, D) over time as shown in figure 5.0. The specific growth rate experiment was done to determine the rate at which SRB regenerates or doubles its original number with respect to time in the presence of the extracts. *Garcinia kola* extract reduced the specific growth rate of SRB significantly while *Anacardium occidentale* extract did not show any appreciable reduction in the specific growth rate of the SRB cultures as shown in table 4 and 5.

Table 1: The growth of SRB during the determination of MIC for *Anacardium occidentale* extract on SRB

Universal bottles	Level of growth	Final conc. of extract in bottles (mg/ml)
B_1-	x	(Chloramphenicol control)
B_2	+	x (Inoculum control)
B_3-	x	(medium control)
B_4	+	3.125
B_5	+	6.25
B_6	+	12.5
B_7	+	25.0
B_8	+	50.0
B_9	+	100.0
B_{10}	+	200.0
Legend: x = (Medium, inoculums and chloramphenicol control)		

+ = Growth observed
- = No growth observed

Table 2: The growth of SRB during the determination of MIC for *Garcinia kola* extract on SRB

Universal bottles	Level of growth	Final conc of extract in bottles (mg/ml)
B_1	- x	(Chloramphenicol control)
B_2	+ x	(Inoculum control)
B_3	- x	(Medium control)
B_4	+	3.125
B_5	+	6.25
B_6	+	12.5
B_7	+	25.0
B_8	-	50.0
B_9	-	100.0
B_{10}	-	200.0

Key: + = Growth observed
- = No growth observed

Table 3: The growth of SRB during the determination of MBC for *Garcinia kola* extract on SRB

Universal bottles	Level of growth	Final conc of extract in bottles (mg/ml)
B_3	-	x (medium control)
B_4	+	3.125
B_5	+	6.25
B_6	+	12.5
B_7	+	25.0
B_8	+	50.0
B_9	-	100.0
B_{10}	-	200.0

Legend: x = (Medium control)
+ = Growth observed
- = No growth observed

Table 4: Specific Growth Rate (Per Hour) of SRB culture inoculated with *Garcinia kola*extract

Days	Bottles			
	A	B	C	D
0	-	-	-	-
3	4.067×10^{-3}	-3.704×10^{-3}	-3.141×10^{-3}	4.810×10^{-3}
6	4.312×10^{-3}	-3.704×10^{-3}	-3.304×10^{-3}	-3.500×10^{-3}

Table 5: Specific Growth Rate (Per Hour) of SRB culture inoculated with *Anacardium occidentale* extract

Days	(Bottles)			
	A	B	C	D
0	-	-	-	-
3	4.107×10^{-3}	3.327×10^{-3}	3.776×10^{-3}	3.711×10^{-3}
6	3.797×10^{-3}	3.007×10^{-3}	3.180×10^{-3}	3.737×10^{-3}

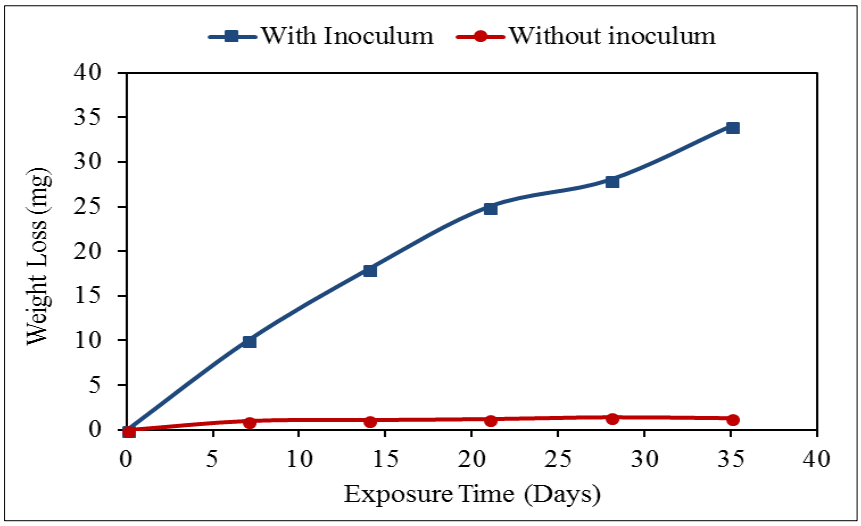

Figure 1.0: Weight loss in metal couponsin SRB environment with respect to time

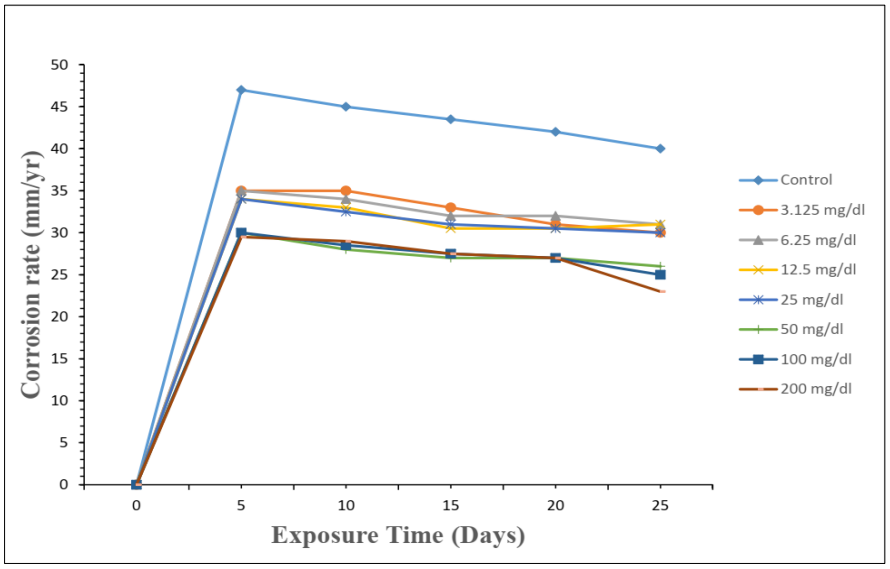

Figure 2.0: Weight loss of metal coupons in SRB environment containing graded concentrations *Anacardium occidentale* extract with respect to time

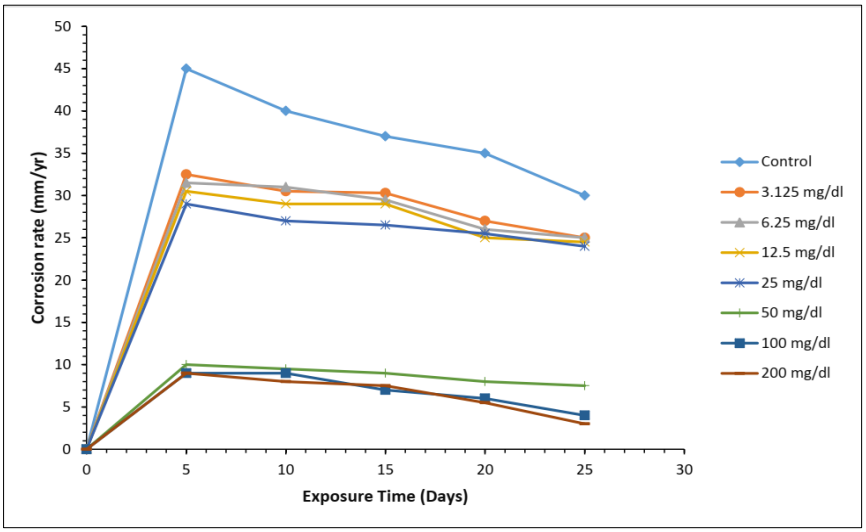

Figure 3.0: Weight loss of metal coupons in SRB environment containing graded concentrationsof *Garcinia kola* extract with respect to time

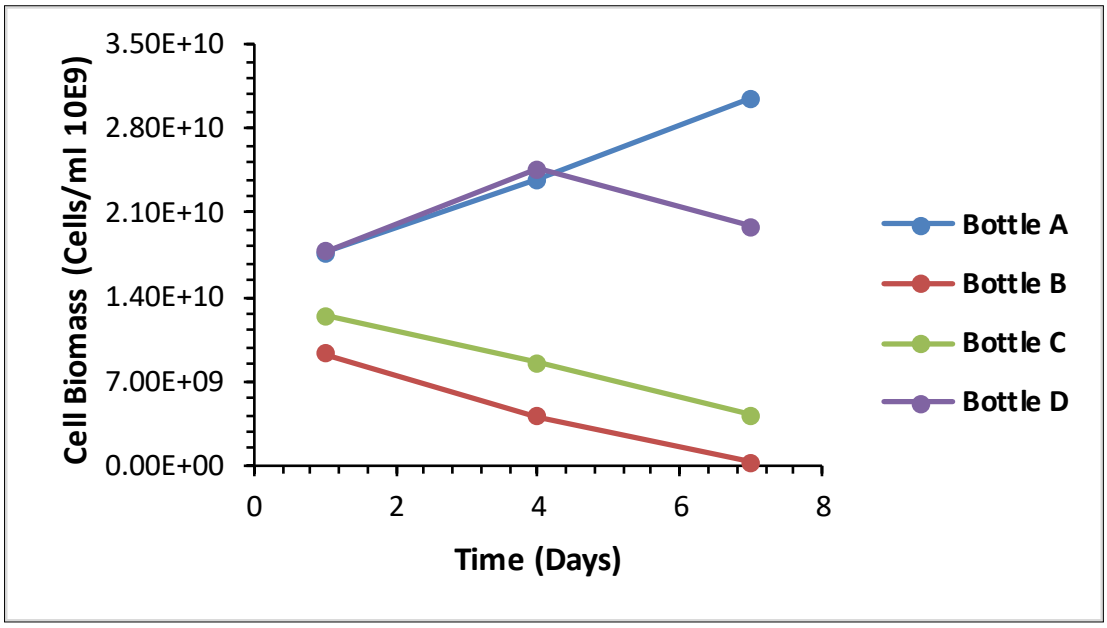

Figure 4.0: Cell biomass of SRB after exposure to *Garcina kola* extract

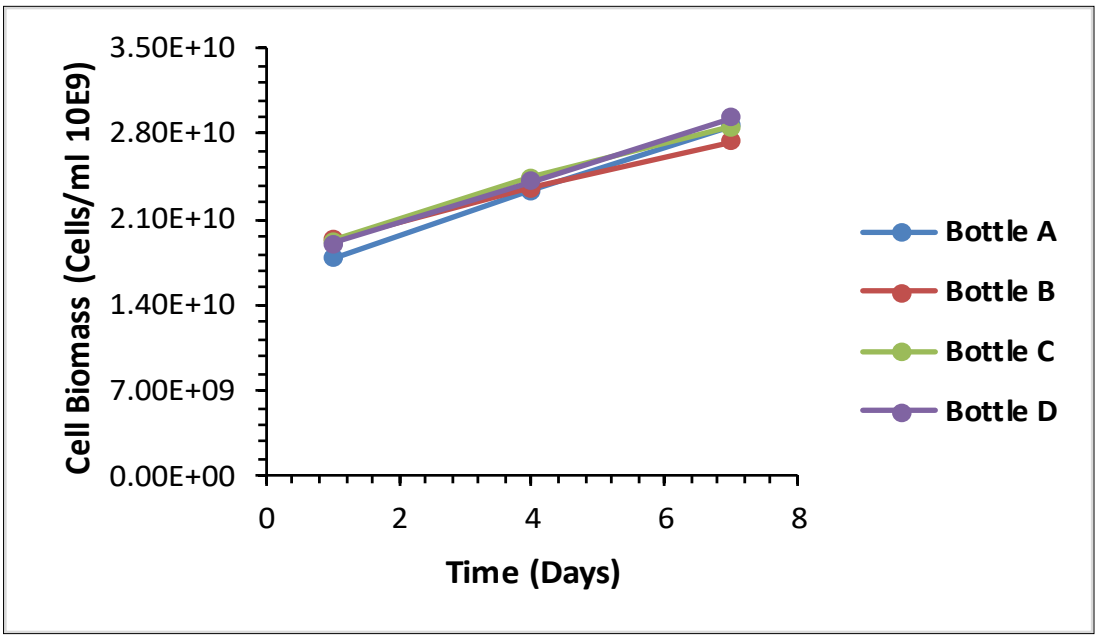

Figure 5.0: Cell biomass of SRB after exposure to *Anacardium occidentale* extract.

4. CONCLUSION

At the ambient working temperature, corrosion inhibition of mild steel coupons was obtained using the extracts of *Garcinia kola*at concentrations above 50mg/ml. The use of *Garcinia kola* for the inhibition of corrosion in the SRB culture medium was effective. The result gotten with the use of *Garcinia kola* extract indicates its viability as a corrosion inhibitor at certain concentrations. This work found reasonable activity at 50mg/ml and above. Further work is ongoing at determining the bioactive compounds that could be responsible for the inhibitory action of the extract.

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