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An Efficient Protocol For *In Vitro* Regeneration of Ginger (*Zingiber Officinale* Roscoe)

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ABSTRACT

Zingiber officinale Roscoe (ginger) is a monocot perennial plant. It is a tropical horticultural plant that is plant valued for its medicinal properties all over the world. It belongs to the Zingiberaceae family and propagated vegetatively and necessitates a large number of rhizomes as starting planting materials. In addition, soil-borne diseases are related to ginger rhizome-based vegetative proliferation. Plant tissue culture techniques have been employed to supply disease-free planting material for a wide range of plant species. Therefore, a study describes an efficient micropropagation protocol was initiated. Surface-sterilized bud rhizomes (explant) with a length of 2-3 cm were treated with ethanol (70%) for 3 minutes, then sodium hypochlorite (2.5%) for 20 minutes, and mercuric chloride (0.1%) for 7 minutes gave 70% survival percentage. Explants were cultured on solid Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzyl adenine (BA), kinetin (Kin), and β - naphthaleneacetic acid, (NAA) to determine the optimal concentration for shoot initiation and multiplication. The maximum mean shoot number (7.05) was obtained in MS medium with 5 mg/l BA, with a shoot length of (4.75 cm) and a leaf number of (7.00). Kin alone or in combination with NAA at 0.5 mg/l, resulted in a lower parameter of shoot multiplication. After four weeks, half-strength MS medium with 2 mg/l indole-3-butyric acid (IBA) significantly increased root number (17.67 roots per shoot), root length (8.17 cm), and plant height (13.33 cm). The rooted plants were transplanted to the greenhouse and planted in pots with sterilized sand and peatmoss in a 1:4 ratio. Acclimatization was successful, with a 73 percent survival rate. After a year in the greenhouse, additional plants sprouted from the rhizome. In conclusion, the current study's final product can be customized to mass-produce disease-free ginger on a massive scale.

KEYWORDS: *Zingiber officinale*; Rhizome buds; ginger; micropropagation.

INTRODUCTION

Medicinal plants have long been used to treat a variety of diseases around the world. One of those plants is *Zingiber officinale* Roscoe, also known as ginger, is a member of the Zingiberaceae family. Ginger is a vital aspect of tropical horticulture and medicine. (Protnoi et al., 2003 and Ravindran and Nirmal, 2005). This plant is a valuable horticultural crop with applications in the pharmaceutical, food, and beverage industries. For thousands of years, it has been used as a well-liked spice and in traditional medicine. (Alachi, 2008 and, Gang and Ma 2008). Ginger rhizomes, both fresh and dried, are used as a spice and herbal medicine all throughout the planet. (Abbas et al., 2011 and David et al., 2016). The essential oil of the plant contains shogaol, gingerol, zingiberene, and bisabolene. In recent years, ginger has been found to possess biological activities, such as antioxidant, anti-inflammatory, and anticancer activities (Nile and Park, 2015, Zhang et al., 2016, Kumar et al., 2014, and Citronberg et al., 2013). In addition, numerous studies have shown that ginger has the ability to prevent and control a variety of disorders, including neurodegenerative diseases, cardiovascular diseases, obesity, diabetes, chemotherapy-induced nausea and emesis, respiratory diseases, digestive, cold and Alzheimer's disease. (Duke et al., 2003, Al- Achi, 2008, Ho et al., 2013, Walstab., 2013, Townsend et al., 2013, Akinyemi et al., 2015, Suk et al., 2017, and Wei et al., 2017). As result of the global COVID-19 virus, ginger was becoming increasingly popular. It aided in the relief of severe symptoms in COVID-19 positive patients and shortened their recovery time (Rangnekar et al., 2020, and Safa et al., 2020). Because of all these diverse advantages of ginger, its demand in global markets is increasing rapidly. Ginger is a sterile plant that does not reproduce. Because its rhizomes are used in vegetative propagation, it cannot be sexually propagated. Using a large proportion of ginger rhizome (60-70% from production), as the rhizome is the most economically exploited section of the plant, it has a detrimental impact on market prices as a raw material for plant cultivation in the next growing season. Apart from that, most diseases, such as bacterial wilt

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(*Pseudomonas solanacearum*), leaf spot or blast (*Phyllosticta zingiberi* and *Pyricularia zingiberi*), microlysis (*Pythium aphanidematum*), Leaf yellowing (*Fusarium oxysporum*), rhizome rot are easily spread through vegetative reproduction via rhizome fragmentation. (Abed-Ashtiani et al., 2016 and kasilingam et al., 2018). In addition, potential for systemic infections by root knot nematodes from propagules is particularly high in a vegetatively propagated plant like ginger. As a result, illnesses are mostly transmitted through rhizome propagules, necessitating the creation of disease-free clones (Shivakumar, 2019). Furthermore, ginger rhizomes are expensive and difficult to handle as planting material because to their bulkiness (Villamor, 2010). Poor seed setting is a significant obstacle to crop improvement in ginger. In addition, the utilization of rhizomes in traditional vegetative propagation enables the plant's genetic characteristics to deteriorate and has low rate of multiplication. To establish large-scale production, it is therefore critical to determine an alternative source of disease-free planting materials. (Hiremath, 2006, and Hasanloo et al., 2014). Therefore, *in vitro* propagation may be a suitable alternative for the effective production of ginger pathogen-free planting material for high-yielding varieties. Also, it's the simplest method for endless source of supply of disease-free planting materials. Several studies have successfully demonstrated the appliance of tissue culture techniques of ginger plant (Abdelmegeed et al., 2011, Mohamed et al., 2011, Kambaska and Santiata, 2009; Miri, 2020 and Zahid et al., 2021). Noteworthy, the initial establishment of a contamination-free culture is difficult due to the exposure of rhizomes to varied soil pathogens (Meenu and Kaushal, 2017 and Thakur et al., 2018). The main purpose of the study was to develop a way for a more rapid and more convenient clonal propagation of ginger during a cost-effective manner for obtaining a large-scale disease-free planting material for off-season and year-round cultivation for the advantage of the farmers.

MATERIAL AND METHODS

Plant material preparation and sterilization

The experiment was conducted within the Tissue Culture Lab at the Desert Research Center from 2018 to 2020. The rhizomes were purchased from Green House Egypt (A company to import herbs). The rhizomes were washed well with water and liquid commercial soap (Pril) several times, followed by the rhizomes being soaked in fungicide (Topsin M) 3g/l for ten minutes, then rinsed with water several times to get rid of the pesticide residues. Washed the rhizomes with running tap water for one hour, followed by the rhizomes were then soaked in Dettol for 30 min with continuous shaking. Finally, the rhizomes were rinsed with running tap water for 1 hour to eliminate any harmful organisms present on the rhizomes.

The buds with a part of the rhizome of 2 to 3 cm are used as explants. The surface sterilization of explants was administered by soaking in 70% ethyl alcohol (v/v) for 3 minutes with continuous shaking, after which they were washed five times with sterile distilled water, followed by 2.5% (w/v) sodium hypochlorite (NaOCl) solution for 20 minutes with continuous shaking, and again with five washes using double distilled water. Then, the use of a sterile solution of mercury chloride ($HgCl_2$) of 0.1% (w/v) and a few drops of Tween-20 for 7 minutes. Finally, the explants were rinsed thoroughly seven times with sterile double distilled water.

Culture medium and condition

The culture medium consisted of Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962; Duchefa, Haarlem, Netherlands) supplemented with 3% (w/v) sucrose and solidified with 0.7% (w/v) agar (BDH Chemicals Ltd., England). The specified of combination of antibiotics, namely streptomycin, and cefotaxime, of 100 mg/l has been added to all media concentrations. Medium without PGRs and antibiotics served as control. Different concentrations of plant growth regulators (Sigma Cell Culture, min. 90% St. Louis, USA) was added to the nutrient medium, according to the growth stage requirements. The pH of the medium was adjusted to 5.8 by adding drops of 0.1 N HCL and 0.1 N NaOH ,then dispensed before autoclaving at 121 °C at a pressure of 1.1 kg/cm^2 for 15 min (Harvey Sterilemax autoclave, Thermo Scientific, USA). Cultures were incubated under an internal temperature of 25 ±2 C°, 70-80% ration relative humidity with a 16:8-h light/dark photoperiod provided by cool white fluorescent lamps (F1 40t9d/38, Toshiba).

Culture establishment and multiplication

For establishment, bud break and multiplication, rhizome buds (explants) were cultured on MS medium supplemented with various concentrations of 6-benzyl adenine (BA) (1, 3, 5, 7 mg/l) and kinetin, N6-furfuryladenine (Kin) (1, 2, 3, 4 mg/l) alone or in combination with 0.5 mg/l β-naphthalene acetic acid (NAA), additionally to PGR-free MS medium was served as control.

Growth percentage, shoots number per explant, shoot length (cm) and number of leaves were recorded after four weeks of culturing. The micropropagation cycle consisted of a monthly subculture.

In vitro rooting

The multiplied shoots (4-5 cm long) were cultured on solid full or half-strength MS medium containing Indole-3-butyric acid (IBA) at different concentrations (1, 2, and 3 mg/l) were used individually or in combination with

(NAA) at 0.5mg/l. Additionally, PGR-free MS medium was used as a control. Root number per shoot, root length (cm) and plant height were recorded after four weeks of culturing.

Acclimatization

The rooted plantlets were carefully removed from the medium and rinsed under running tap water to eliminate any remaining agar. The roots were soaked in a fungal disinfectant (Rizolex 1g/l) for 5 minutes and transferred to 6 cm diameter plastic pots, filled with a sterilized sand and peat moss in a 1:4 ratio. Plantlets were covered with transparent white plastic and transferred to greenhouse at 28±2 C° and 70-80% relative humidity under shading to maintain humidity. After four weeks, Plastic bags were gradually removed from pots for proper hardening, and plantlets were irrigated with ¼ MS medium every 3 days for 8 weeks. Finally, they were transferred to the plantlets outside the greenhouse and then they were irrigated with normal water. For eighteen months, the plants were monitored in and out of the greenhouse.

Experimental design and statistical analysis

The experimental data was conducted using a completely randomized design, with 20 replicates for each treatment. ANOVA, a statistical analysis program, was used to perform data variance analysis. The difference in mean between treatments was tested for significance at the 5% level using Duncan's multiple range test. Duncan (1955), with modifications by Snedecor and Cochran (1990).

RESULTS AND DISCUSSION

In general, contamination is a determinant of *in vitro* propagation, particularly in rhizomes, which grow under the surface of soil and are consequently exposed to infection by fungi and bacteria, which threaten *in vitro* culture, where the rhizome is utilized as an explant (Figure 1A, and B). So, it was used streptomycin, and cefotaxime in the medium to reduce the amount of contamination. 70% of aseptic cultures were obtained following the sterilization procedure described, while 30% of explants were browned or infected (Figure 1 C).

Figure 1. (A) Rhizomes **(B)** Rhizome bud (explant) **(C)** Contaminated rhizome bud

Effect of growth regulators on initiation and multiplication

We investigated the effect of PGRs on growth percentage, shoot number per explant, shoot length (cm), and leaves number using a PGR-free medium as a control. The presence of PGRs is critical for growth percentage in *Z. officinale*. With the exception of the control, all of the tested treatments sprouted from the rhizome bud after one week, of culture. Figure 2 (A, B, C) shows bud initiation of *Z. officinal* after one week, two weeks and three weeks, respectively.

Figure 2. Sprouting of rhizome bud of *Z. officinale*.
A. After one week from culturing.
B. After two weeks from culturing.
C. After three weeks from culturing.

The influence of PGRs on growth percentage, shoots number/explant, shoot length (cm), and leaves number is summarized in Table 1. The maximum growth percentage (80.00%), shoot number per explant (7.05), shoot length (4.32 cm), and leaves number (7) were seen in explants cultured on medium supplemented with 5 mg/l BA (Figure 3. A, B and C).

Table: 1 Effect of different growth regulators on growth percentage, shoots number, length and leaves number of *Zingiber officinale* Roscoe

Growth regulators (mg/l)			Growth (%)	Shoots no/explant	Shoot length (cm)	Leaves no/shoot
BA	kin	NAA				
0.0	0.0	0.0	0.0 h	0.0 i	0.0 h	0.0 h
1	0.0	0.0	46.70 d	3.61 d	2.21 g	5.33 d
3	0.0	0.0	53.30 c	3.95 d	3.06 e	6.67 b
5	0.0	0.0	80.00 a	7.05 a	4.32 a	7.00 a
7	0.0	0.0	73.33 b	2.57 b	4.75 b	6.00 c
1	0.0	0.5	33.31 f	2.57 f	3.60 d	4.33 e
3	0.0	0.5	40.00 e	3.72 d	4.03 c	5.33 d
5	0.0	0.5	46.70 d	4.05 c	3.50 d	6.33 c
7	0.0	0.5	73.33 b	5.10 b	3.80 d	6.00 c
0.0	1.0	0.0	20.00 g	0.7 h	2.00 g	3.00 f
0.0	2.0	0.0	33.31 f	1.63 g	2.70 f	3.67 f
0.0	3.0	0.0	33.31 f	3.27 e	2.50 f	2.67 g
0.0	4.0	0.0	40.00 e	3.83 d	2.10 g	4.33 e
0.0	1.0	0.5	33.31 f	0.94 h	2.90 f	3.67 f
0.0	2.0	0.5	33.31 f	0.98 h	3.10 e	2.67 g
0.0	3.0	0.5	40.00 e	1.76 g	4.50 a	4.00 e
0.0	4.0	0.5	46.70 d	2.14 f	4.90 a	3.67 f

Means following by the same within a column are not significantly different at $p \leq 0.5$

It is well known that cell division, shoot multiplication and axillary bud formation can be promoted by the cytokinin. BA had a considerable effect on *Z. officinale* multiplication. Whereas, Kin, alone or in combination with NAA, resulted with decreased growth in all parameters.

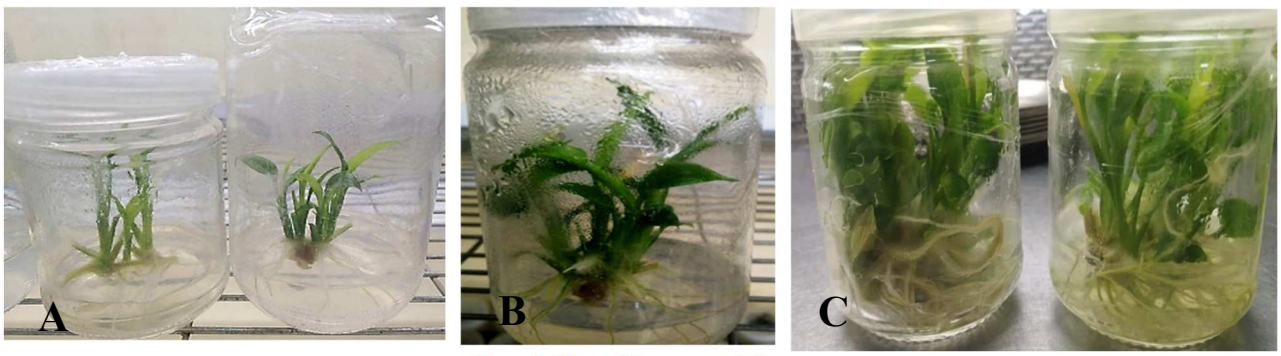

Figure 3. Shoot multiplication of *Z. officinale* on MS medium containing BA at 5 mg/l
A. After four weeks of culture. **B.** After five weeks of culture. **C.** After six weeks of culture.

In all growth measurements, kinetin alone or in combination with NAA produced lower values. With the exception of kin. at 3 and 4 mg/l plus NAA at 0.5 mg/l gave the longest shoot length (4.5 and 4.9 cm, respectively) and there was no significant difference between them and BA at 5 mg/l. (Rout et al., 2001; Hiremath 2006; Kavyashree 2009; Kambaska and Santilata 2009; Abbas et al., 2011; Ayenew et al., 2012; and Miri, 2020) have all reported that BA has a stimulatory effect on multiple shoot development in *Z. officinale*. This could be due to its potential to stimulate morphogenesis induction by metabolizing or generating endogenous hormones (Ahmed and Anis, 2014). However, increasing the concentration of BA further to 5 mg/l decrease growth percentage, shoot number, shoot length and leave number. This observation is supported by Jafari et al., 2011; Sathyagowri and Seran, 2011 who found that high concentration of BA was not essential for shoot multiplication due to the reduction in the shoots number of ginger. This is explained by the high concentration of cytokinin lead to exogenous application of cytokinin or the expression of isopentenyl transfer (IPT) gen increases the endogenous cytokinin level in the cell, which increases the cytokinin oxidase activity and, consequently, down-regulates the elevated cytokinin in the plant cells (Motyka et al., 2003 and Wang et al., 2014).

***In vitro* rooting**

For successful *in vitro* propagation of plants, rooting of shoot is an important step to adapt to the survival and growth of regenerates plants in acclimatization (Krupa-Małkiewicz and Mgłosiek 2016). The addition of auxins to in vitro cultures hasten up the production of roots and increases the rate of rooting (Martins et al. 2013; Miri and Roughani 2018). With the shoot multiplication stage of ginger, roots naturally produced. This is consistent with previous observations that root induction happened naturally as a result of ginger shoot multiplication (Sathyagowri and Seran, 2011 and Zuraida et al., 2016). Although roots were naturally generated in all treatments, they were insufficient to transplant the plantlets in the case of separating multiple shoots. This may be due to the endogenous concentration of IAA in ginger shoots moving to the lower part and its concentration increasing. So that, stimulate the root at the base of the bud or provide proper hormonal balance, and improve rooting with other exogenous auxins (Martins et al., 2013). To transplant and survive the *in vitro*-raised ginger plantlets, an adequate number of roots per plantlet was required. In order to investigate different types and concentrations of auxins for *in vitro* rooting of ginger, a separate experiment was done. Table 2 shows the concentrations of the two auxins (IBA and NAA) and two strength of MS medium tested for rooting of *Z. officinale*. The highest root numbers (17.67), root length (8.17 cm) and plant height (13.33 cm) were achieved in a half-strength MS medium fortified with 2 mg/l IBA after four weeks of culture (Figure 4). This finding is consistent with some studies (Bhagyalakshmi and Singh 1988; Sumon et al., 2019 and Miri, 2020) that found IBA at 2 mg/l to be more stimulating to ginger root growth. On the other hand, it was found NAA to be more effective than IBA for *in vitro* root induction of ginger (Kambaska and Santilata 2009 and Abbas, et al., 2011 and Zahid et al., 2021).

Table 2: Effect of strength MS medium supplemented with different concentration of auxin (IBA and NAA) on *in vitro* rooting of *Zingiber officinale* after four weeks.

Medium strength	Auxin concentration mg/l		Root numbers/ shoot	Root length (cm)	Plant height (cm)
	IBA	NAA			
Agar+ Sugar	0.0	0.0	5.67 g	5.30 c	6.33 g
Half MS	1.0	0.0	8.67 d	5.96 b	10.50 c
Full MS	1.0	0.0	6.33 f	6.46 b	11.33 b
Half MS	2.0	0.0	17.67 a	8.17 a	13.33 a
Full MS	2.0	0.0	9.33 c	6.00 b	11.00
Half MS	3.0	0.0	10.33 b	5.77 c	10.50 c
Full MS	3.0	0.0	7.67 e	4.10 d	8.50 e
Half MS	0.0	1.0	6.67 f	4.37 d	7.33 f
Full MS	0.0	1.0	9.33 c	3.50 e	9.50 d
Half MS	0.0	2.0	8.33 d	5.96 b	6.50 g
Full MS	0.0	2.0	7.33 e	5.17 c	9.33 d
Half MS	0.0	3.0	6.67 f	5.67 c	9.66 d
Full MS	0.0	3.0	6.67 f	4.67 d	7.23 f

Means following by the same within a column are not significantly different at $p \leq 0.5$

Figure 4. Rooted plantlet of *Zingiber Officinale* on half-strength MS medium containing 2 mg/l IBA

It has also been reported that IBA induced lateral rooting, (Spethmann and Hamzah, 1988; Riov, 1993; De Klerk, 1999 and Ludwig-Muller, 2000). Furthermore, IBA is more stable and less sensitive to auxin degrading enzymes. (Epstein and. Ludwig-Muller, 1993 and Riov, 1993). In general, half-strength nutrient medium increased overall root growth when compared to full strength nutrient medium salts for *Z. officinale* root formation. This is consistent with previous findings Abbas et al. (2010) revealed that half-strength basal salts media were more beneficial for root development in Taxus species than full-strength basal salts media.

Acclimatization

Acclimatization is the last phase of micropropagation when plants produced *in vitro* are gradually adjusted to external conditions. Various conditions *in vitro* may result in development of plants with altered morphology, anatomy, and physiology. A transfer to external environment may result in damage and physiological disorders. Usually, stomata of *in vitro*-generated plants do not function normally, so a reduction of the transpiration rate is crucial for plant hardening. Therefore, the acclimatization stage is considered one of the most important stages of *in vitro* propagation. In a greenhouse under shade.

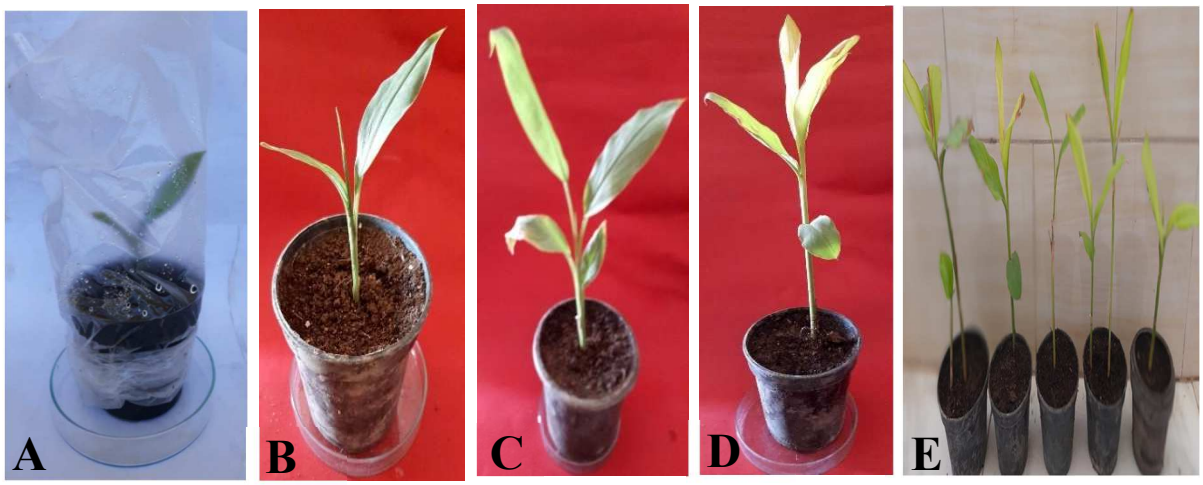

Figure 5. Acclimatization plantlets of *Z. officinale*, under greenhouse conditions **(A)** one week old of transferring, **(B)** four weeks old plant, **(C)** and **(D)** after five weeks old of transferring, **(E)** two months old plant.

Ginger *in vitro*-rooted plantlets were effectively acclimatized at an 73 percent survival rate in sand and peatmoss 1:4 (v/v) (Figure 5 A, B, C, D, E). After five months, the acclimatized plants were released outside the greenhouse. Burning within the edges of some leaves, may be the result of direct sun exposure or extreme heat, and we solved the problem by putting it under the shade of some trees, and followed it up with irrigation and fertilization at a rate of one each 3 days (Figure 6 A, B and C). Every morning and evening, the plants are misted with water to maintain the humidity around them. After ten months, the plant was ready to produce new plants, which appeared in the

same pot. This indicates that the plant has a strong root, capable of producing rhizomes and therefore new plants, as shown in. Figure 7, (A) shows the length and strong of the roots, as well as the emergence of new plants and (B), The plants are 12 months old and (C) the plants outside the greenhouse after 18 months of acclimatization.

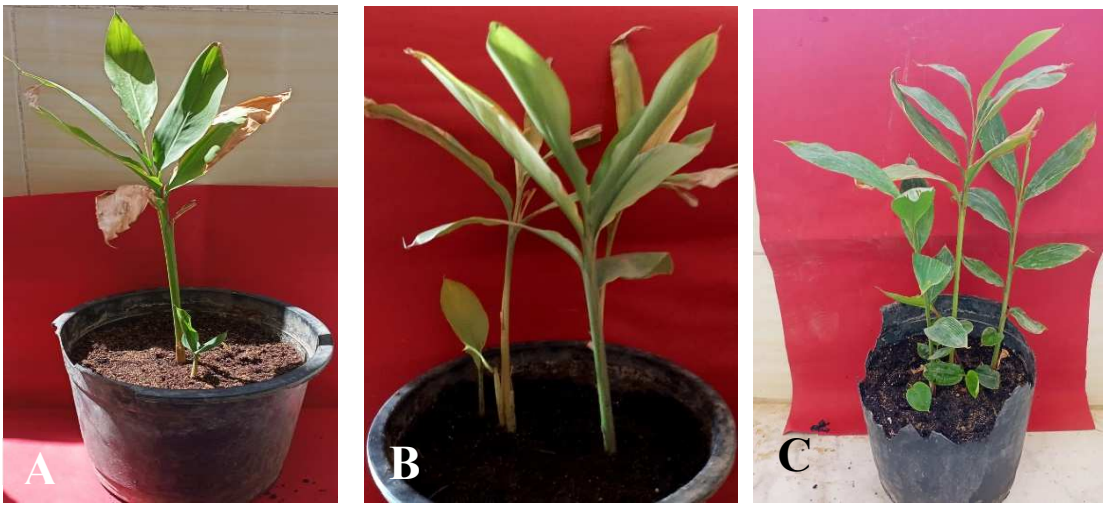

Figure 6. Acclimatized plants outside the greenhouse, **(A)** after five months, **(B)** after seven months, **(C)** after ten months

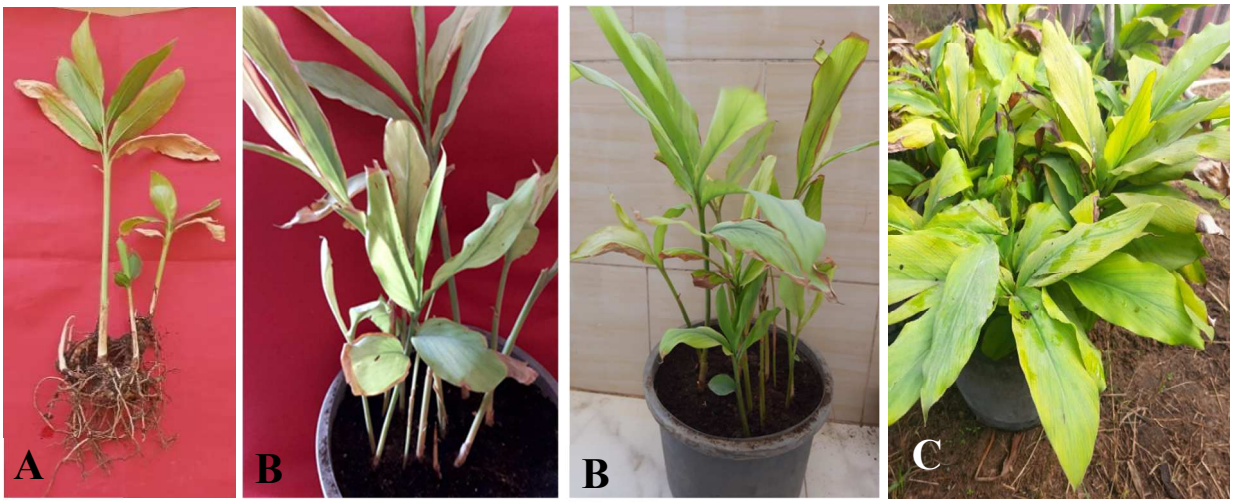

Figure7. **(A)**Strong and tall roots and emergence some plants grown outside greenhouse **(B)** twelve months old plants **(C)** eighteen months old plants

CONCLUSION

The conclusions of this study can be described as a promising and successful ginger *in vitro* propagation strategy. The utilization of rhizome buds allows for some sterilizing and contamination minimization because it is one of the determining variables for the effectiveness of micropropagation. The highest shoot multiplication was obtained when the culture was carried out on MS medium supplemented with 5 mg/l benzyl adenine (BA). In this study, indole butyric acid (IBA) was found to be superior to β-naphthalene acetic acid (NAA) for root establishment. A remarkable commercial offer is rapid micropropagation.

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Evaluation of the Ameliorative Roles of Vitamins A, C and E on Haematological Parameters of *Clarias Gariepinus* (Burchell, 1822) Fingerlings Exposed to Cadmium Chloride

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ABSTRACT

The ever increasing anthropogenic activities all over the world that usually lead to the release of myriads of pollutants such as cadmium call for concern. In the present study, the effects of cadmium chloride on the haematology of *C. gariepinus* and how such effects can be mitigated through the administration of vitamins were investigated. *C. gariepinus* fingerlings (whose initial weight ranged from 3-11g, standard length ranged from 7.9-9.4cm and total length ranged from 8.9-10.9cm) were exposed to sub-lethal concentrations of Cd (00, 12mg/L, 16mg/L, 20mg/L and 24mg/L) with replicate in each case. 12mg/L each of the vitamins were administered across all the bud. Fresh concentrations of both toxicants and vitamins were administered every 72 hours for a period of 12 weeks every time the water medium was changed. The various treatments group include Cd (Cd only), CdVA (Cd+vitamin A), CdVC (Cd+vitamin C) and CdVE (Cd+vitamin E) with T1-T4 and replicate in each case. 3 samples of the fish were randomly selected and sacrificed from each aquarium tank every 4th week of the exposure period. The blood collected were analyzed for White Blood Cell count (WBC), Red Blood Cells (RBC), Haemoglobin Concentration (HGB), Pack Cell Volume (PCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin Concentration (MCHC) and Platelet Count (PLT). The data generated were subject to one way analysis of variance at $P \leq 0.05$. The results indicate that in samples exposed to Cd only treatments for a period of four weeks, there were increased values of production of WBC in all treatments when compared to the control. There were decreases in the production values of PLT in all treatments when compared to higher values obtained in the control samples. The RBC, PCV and PLT mean values in the control are significantly higher than other treatments. After the 8th week of exposure, the WBC, RBC, and PCV mean values in the control are significantly higher than other treatments. MCHC and PLT mean values in the control are significantly different from other treatments. After the 12th week of exposure, RBC, Hb, PCV, MCV, MCH, MCHC and PLT mean values in T1 are significantly lower than other treatments. In CdVA treatments in the 4th week of exposure indicated an increased production of WBC in all treatments; increased values of PLT were obtained in all treatments. The PCV and MCHC mean values in T1 are significantly different from other treatments. The mean values of the blood PLT are all significant with higher significance in T1 and T3. After the 8th week of exposure, all the parameters are not significantly different from each other. Similarly, at the 12th week of exposure there is no significant difference in all treatments. Level of production of blood parameters was generally low in all treatments. In the case of samples exposed to CdVC for a period of four weeks, there were increases in WBC and PLT values in all treatments. After the 8th week of exposure, mean values of all the parameters have no significant differences. At the 12th week of exposure, there were also no significant differences in the mean values of all the parameters. Samples exposed to CdVE treatments after four weeks, displayed higher values of WBC, slightly lower values of RBC, Hb and PCV in all treatments. There were marked increases in the production values of blood PLT in all treatments. The MCH and PCV mean values in T2 were significantly higher than in other treatments. After 8 weeks of exposure, the WBC mean values in T4 were significantly higher than other treatments. At the end of the 12th week, WBC and RBC mean values in T2 and T1, respectively were significantly higher than other treatments. The vitamins supplemented treatments displayed varying levels of ameliorations far better than the Cd only group. Amongst these, the CdVC and CdVE treatment groups fared better than others. The outcome of this research has shown the impacts of vitamins A, C and E in mitigating the effects of the toxicant and can serve as remedy in heavy metal toxication when appropriate concentrations are administered.

KEY WORDS: Cd toxicant, ameliorative roles, haematological parameters, vitamin supplements, Cd treatment groups and *Clarias gariepinus*

1.0 INTRODUCTION

Fish is a rich source of animal protein throughout the world. Due to its nutritional value (Tingman *et al*., 2010), the demand for fish food has been on the increase with the increasing human population (FAO 2010, 2012). African catfish, *Clarias gariepinus* is an important commercial fish due to its high growth rate, high consumer acceptability, and ability to withstand poor water quality, and oxygen depletion (Adewolu *et al*., 2008; Karami *et al*., 2010). Fishes serve as early warning indicators of pollution in the aquatic systems and can be considered to be the

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most standard choice as test organisms because they are the best understood organism in the aquatic environment and its importance to man (and other organisms) as a source of protein (Murtala *et al*., 2012). The presence of toxicants in the environment of organisms has myriads of effects on fish physiology. The haematological profile of a species is a good indicator of the levels of environmental stress and, consequently, changes in blood parameters have been used to assess the effects of environmental pollution (Vinodhini and Narayanan, 2009).

The presence of pollutants in the environment of an aquatic organism such as fish can lead to the production of reactive oxygen species and consequently, oxidative stress. Heavy metals could be essential or non-essential. Heavy metals such as Fe, Cu, Zn, Ni, Co, Cr, and Mn are vital to humans only at lower concentrations, but they become more toxic when they are taken up more than the bio-recommended limits (Shilpi *et al*., 2015). It is also known that even essential metals may be toxic to the biological activities of organisms above certain concentrations (Merciai *et al*., 2014). Heavy metals such as lead, mercury, cadmium, etc., naturally occur in the deep layers of the earth and are present in the soils, rocks and sediments with high concentrations (Waheed *et al*., 2020). The ability of heavy metals to bioaccumulate and biomagnifying and difficult to be eliminated from the body by the ordinary metabolic activities make them one of the most dangerous sources of chemical water pollution to fish, causing big losses to fish and effects on the fish consumers (Mirghaed *et al*., 2018). Heavy metals are known to elicit oxidative stress in organisms when the threshold is exceeded. Heavy metals are also known to promote oxidative damage by increasing the cellular concentration of reactive oxygen species (ROS) in fish, consequently, a response of antioxidative defences (Monteiro *et al*., 2010). Cadmium, which is a non-essential element with no biological role, is toxic even at low levels. Heavy metals induce significant damage to the physiologic and biochemical processes of the fish and subsequently to fish consumers (Mehana *et al*., 2020). Unlike essential elements that are required in the diet for optimal growth, functioning and sustenance of the internal environment (Isibor and Imoobe, 2017); the presence of Cd is entirely deleterious. Among all the heavy metals, Cd, arsenic, mercury and lead pose the highest degree of toxicity and that is of great concern to plants and human health (Athar *et al*., 2018). They can also be classified as carcinogens (Chung *et al*., 2016).

Vitamins C and E supplementations have been reported to play a positive role in detoxification of mercury toxicity especially at lower concentrations (Thakur and Kanshere, 2014). Likewise, it has also been demonstrated that Cd-induced changes were significantly improved with supplementation of vitamin E as well as tomato paste (Mekkawy *et al*., 2013). Ascorbic acid is well known for its antioxidant activity, acting as a reducing agent to reverse oxidation in liquids (McGregor and Biesalski, 2006). The main biological function of vitamin E is its direct influence on cellular responses to oxidative stress through modulation of signal transduction pathway (Pratt *et al*., 2010). Vitamins E and C supplementation can induce protective effects on certain conditions after free radical-mediated cellular damage or disruption (Yolanda and Maria, 2012). $CdCl_2$ increases TLC (Total leukocytes count) and decreases Hb content as compared to control; and the exposure of heavy metal with ascorbic acid led to a decrease in the TLC and increase in Hb contents as compared to those of heavy metal intoxicated fishes (Borane, 2013). In addition, Vitamin C has potent antioxidant activity against cadmium and mercury sensitive haematological parameters (Hounkpatin *et al*., 2012). The blood parameters are usually affected in one way or the other in the presence of toxicants. For instance, Hounpaktin *et al*. (2012) demonstrated a significant decrease in white and red blood cell count, reduced hemoglobin and mean corpuscular concentrations when high concentrations of mercury and the combination of high concentrations of cadmium and mercury were administered. However, co-administration of mercury, cadmium and mercury and vitamin C had a protective effect on the harmful metals. The values of the haematological parameters were also increased due to treatment with vitamin C.

Changes in the haematological and genotoxic components of cat fishes have been reported from the field and laboratory researches (Guedenon *et al*., 2012; Bolognesi and Cirillo., 2014; Singh *et al*., 2017) but there is paucity of information on the effects of specific toxicants such as Cd and what happens when supplemented with vitamins. This is why this study attempted to bridge the gap in knowledge on the haematological effects of sub-lethal concentrations of cadmium toxicant and how vitamins A, C and E supplements can ameliorate such effects on *C. gariepinus* fingerlings.

2.0 MATERIALS AND METHODS

2.1 Samples/materials collection and Acclimatization

A total number of four hundred (400) fingerlings of *C. gariepinus* were purchased from a commercial fish farmer and transported in 50L containers filled with water to the Old Farm Research Unit of the Department of Water, Aquaculture and Fisheries Technology, Bosso Campus, Federal University of Technology, Minna, Nigeria. The fishes were placed in fish ponds with water for acclimatization. The fishes were fed twice daily (morning and

evening) with Blue Crown feed (3mm) for 14 days (2 weeks) for acclimatization. The holding water was changed every 3 days during the period.

The vitamins A, C and E granules or pellets were purchased from commercial chemical stores. About 500g units of the granules in each case were used as the supplements in percentages corresponding to the sub-lethal concentrations of the treatments. The toxicant, Cd (2pieces of 100g) analar grades were purchased from commercial chemical stores and stored in a cool dry condition throughout the period of the experiment. These toxicants were administered according to the concentrations and the sub-lethal concentrations corresponding to the sub-lethal concentrations of the treatments during the chronic phase of the exposure.

2.2 Experimental Set up

Five (5) treatments including control with two replicates in each treatment were set up for the Cd, Vitamins A, C and E; and the sub-lethal exposures were run for a period of twelve (12) weeks. Sampling was made from each trough randomly by picking out 3 samples every four (4) weeks for the haematological parameters. Sub-lethal concentrations of cadmium chloride ($CdCl_2$) used for the chronic exposure were 12mg/L, 16mg/L, 20mg/L and 24mg/L as T1-T4 respectively; each treatment was in two replicates containing 12 fish in 20L plastic aquaria for the Cd, Vitamins A, C and E supplemented exposures. The minimum concentration of the toxicant serves the same concentration of the vitamins. The water was changed and fresh toxicants and vitamins with the same set of concentrations were added every 72 hours according to Organization for Economic Co-operation and Development (OECD, 2007) standards.

2.3 Determination of Haematological Parameters of *C. gariepinus* exposed to sub-lethal concentration of cadmium

Blood samples were collected three times on a monthly basis (once every 4 weeks) from each sample and replicated. The blood was collected by inserting the sterile syringe between the operculum and the pectoral fin on the ventral side of the fish; and then drawn by creating suction pressure that allowed easy flow of the blood into the syringe. This method gave more blood with ease than drawing them from the caudal vein of the fish. White Blood Cell count (WBC), Red Blood Cells (RBC), Haemoglobin Concentration (HGB), Pack Cell Volume (PCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin Concentration (MCHC) and the Platelet Count (PLT) of the blood collected from the samples of each treatment and replicate with 1ml heparinized sterile syringe into EDTA test tubes containing little quantity of anti-coagulant (Abdulkareem *et al*., 2017; Usman *et al*., 2019), were determined in the Medical Laboratory Services of Minna General Hospital, Niger State**.** These parameters were determined using Mindray (BC-5300) Auto Hematology Analyzer for full blood count. This works on the principle of laser scatter, flow cytometry and chemical dye to provide reliable and accurate 5-part differentiation on blood cells.

2.4 Data Analysis

The blood parameters of the samples exposed to sub-lethal concentrations of the toxicants as well as those treatments supplemented with vitamins were analyzed using One Way Analysis of Variance followed by Duncan Multiple Range Test to separate the means where significant at $P \leq 0.05$ level of significance using SPSS Statistical Package (version 20.0 for Windows).

3.0 RESULTS AND DISCUSSIONS

3.1 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd toxicants and the respective supplemented treatments with Vitamins A, C and E for a period of four, eight and twelve weeks

In samples exposed to Cd only treatments for a period of four weeks, there were increased values of production of white blood cells counts (WBC) in all treatments when compared to the control. There were decreases in the production values of blood platelets (PLT) in all treatments when compared to higher values obtained in the control samples. In like manner, after the eight weeks of exposure, there were increased values (higher than what were obtained after four weeks of exposure) of WBC in all treatments when compared to the control. There were also decreased values of RBC, Hb and PCV, MCHC in all treatments when compared to the control group. Drastic reductions in values of PLT in all treatments when compared to the control were also obtained. Furthermore, there were increased values of WBC after twelve weeks of exposure in samples that survive till the end. Decreased values of RBC, Hb, PCV, MCV, MCH, MCHC and PLT were also recorded in samples that survive to the end when compared to the control. From the statistical analysis, the WBC mean values in T1-T4 after the 4^{th} week of exposure are significantly higher than the control. While the Hb mean values of the T1-T4 are significantly lower than the

control. On the other hand, the RBC, PCV and PLT mean values in the control are significantly higher than those of T1-T4. MCV mean values in T2 are significantly higher than in other treatments. Likewise, the MCHC mean values in control and T1 are significantly higher than T2, T3 and T4 values. However, after the 8th week of exposure, the WBC, RBC, and PCV mean values in the control are significantly higher than T1-T4 mean values. MCHC and PLT mean values in the control are significantly different from other treatments. Meanwhile, after the 12th week of exposure, RBC, Hb, PCV, MCV, MCH, MCHC and PLT mean values in other treatments are significantly higher than T1. After the 8th week there were high mortality rates especially in the higher concentrations (Tables 3.1- 3.3).

Table 3.1 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd for a period of four weeks

Parameters	Treatments				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	9.80±0.12^a	18.00±0.58^c	18.50±0.29^c	17.00±0.58^b	18.00±0.58^c
RBC (Mil/mm^3)	3.60±0.06^c	2.80±0.00^b	2.45±0.03^a	2.75±0.03^b	2.80±0.03^b
Hb (g/dl)	10.05±0.14^b	7.60±0.46^a	7.65±0.26^a	7.40±0.35^a	7.30±0.17^a
PCV (%)	29.50±0.29^b	22.50±1.15^{ab}	22.50±0.87^{ab}	22.00±1.15^a	21.50±0.29^a
MCV (Fl)	81.50±2.02^{ab}	78.00±4.04^a	92.00±4.62^b	79.50±4.91^{ab}	77.00±2.89^a
MCH(Pg)	27.00±0.58^a	27.00±1.73^a	30.50±1.44^b	26.50±1.44^a	26.00±1.15^a
MCHC (g/dl)	33.50±0.29^b	34.50±0.29^b	29.50±0.29^a	29.50±0.29^a	29.00±0.58^a
PLT (Cmm)	206.00±1.15^b	112.00±2.89^a	111.50±0.87^a	114.00±2.89^a	110.50±2.02^a

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤0.05.

Table 3.2 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd for a period of eight weeks

Parameters	Treatments				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	9.40±0.40^a	18.50±0.50^{bc}	18.00±1.00^b	17.00±1.00^b	20.00±1.00^c
RBC (Mil/mm^3)	3.60±0.20^b	2.60±0.30^a	2.30±0.50^a	2.65±0.15^a	2.60±0.20^a
Hb (g/dl)	10.45±0.05^c	8.00±0.70^b	6.75±1.05^a	7.35±0.25^{ab}	6.65±0.85^a
PCV (%)	31.00±0.00^b	23.50±2.50^{ab}	20.00±3.00^a	22.00±1.00^a	19.50±2.50^a
MCV (Fl)	86.50±4.50^c	92.50±20.50^d	88.00±6.00^c	83.00±1.00^b	74.50±3.50^a
MCH (Pg)	28.50±2.50^b	31.00±6.00^c	29.00±2.00^{bc}	27.50±0.50^b	25.00±1.00^a
MCHC (g/dl)	32.60±3.00^c	29.00±1.00^a	29.00±0.00^a	30.00±0.00^{ab}	29.50±0.50^{ab}
PLT (Cmm)	227.50±7.79^c	110.50±6.50^a	117.50±10.50^{ab}	127.00±1.00^b	124.00±1.00^{ab}

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤ 0.05.

Table 3.3 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd for a period of twelve weeks

Parameters	Treatments				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	8.00±0.00^a	12.00±12.40^b	0.00±0.00	0.00±0.00	0.00±0.00
RBC (Mil/mm^3)	3.45±0.26^b	1.47±1.28^a	0.00±0.00	0.00±0.00	0.00±0.00
Hb (g/dl)	11.50±0.60^b	3.87±3.35^a	0.00±0.00	0.00±0.00	0.00±0.00
PCV (%)	34.50±1.60^b	11.30±9.82^a	0.00±0.00	0.00±0.00	0.00±0.00
MCV (Fl)	100.50±11.60^b	51.33±44.46^a	0.00±0.00	0.00±0.00	0.00±0.00
MCH (Pg)	34.50±5.60^b	17.33±15.03^a	0.00±0.00	0.00±0.00	0.00±0.00
MCHC (g/dl)	30.00±0.00^b	17.33±15.02^a	0.00±0.00	0.00±0.00	0.00±0.00
PLT (Cmm)	247.00±7.79^b	77.33±66.98^a	0.00±0.00	0.00±0.00	0.00±0.00

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤0.05.

In another development, CdVA treatments in the first four weeks of exposure indicated an increased production of WBC in all treatments. Similarly, increased values of PLT were obtained in all treatments. On the other hand, samples exposed for eight weeks indicated that there were increased production values of WBC, MCV

and MCHC; lower values of RBC in samples that survived to this stage. Moreover, samples exposed for a period of twelve weeks also displayed increased values of WBC but decreased values of RBC, Hb, PCV, MCV, MCH, MCHC and PLT in samples that survived to the end. From the statistical analysis in CdVA mean values after the 4th week of exposure, the PCV mean values are significantly different in descending order from T1-T4. MCV and MCH mean values are significantly higher in T2 than in other treatments. The MCHC mean values are significantly higher in T1 than in other treatments. The mean values of the blood PLT are all significant with higher significance in T1 and T3. In addition to these, after the 8th week of exposure, all the parameters were not significantly different from each other. Similarly, at the 12th week of exposure there is no significant difference in all treatments. The level of production of blood parameters was generally low in all treatments. (Tables 3.4 - 3.6).

Table 3.4 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd supplemented with vitamin A for a period of four weeks

Parameters	Treatments				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	9.80±0.12^{a}	13.00±0.00^{bc}	14.00±0.58^{c}	12.00±0.58^{b}	14.00±1.15^{c}
RBC (Mil/mm^3)	3.60±0.06^{b}	3.00±0.00^{a}	2.95±0.09^{a}	3.05±0.03^{a}	3.05±0.03^{a}
Hb (g/dl)	10.05±0.14^{c}	8.00±0.06^{a}	9.10±0.06^{b}	8.65±0.14^{a}	8.20±0.12^{a}
PCV (%)	29.50±0.29^{c}	23.50±0.29^{a}	27.00±0.00^{d}	25.50±0.29^{c}	24.50±0.29^{b}
MCV (Fl)	81.50±2.02^{b}	78.00±1.15^{a}	91.50±2.60^{d}	83.00±0.00^{c}	81.50±0.87^{b}
MCH (Pg)	27.00±0.58^{a}	26.50±0.29^{a}	30.50±0.87^{b}	28.00±0.00^{a}	27.00±0.58^{a}
MCHC (g/dl)	33.50±0.29^{b}	34.00±0.00^{b}	29.00±0.00^{a}	29.00±0.00^{a}	29.50±0.29^{a}
PLT (Cmm)	206.00±1.15^{a}	228.00±3.46^{d}	216.00±0.58^{b}	239.00±4.62^{e}	224.50±1.44^{c}

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤0.05.

Table 3.5 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd supplemented with vitamin A for a period of eight weeks

Parameters	Treatments				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	9.40±0.23^{b}	0.00±0.00	6.67±3.33^{a}	8.67±4.33^{b}	8.67±4.33^{b}
RBC (Mil/mm^3)	3.60±0.12^{c}	0.00±0.00	2.13±1.07^{b}	2.07±1.03^{b}	1.67±0.83^{a}
Hb (g/dl)	10.45±0.03^{b}	0.00±0.00	7.00±3.50^{a}	6.67±3.33^{a}	6.47±3.23^{a}
PCV (%)	31.00±0.00^{c}	0.00±0.00	20.67±10.33^{b}	20.00±10.00^{a}	19.33±9.67^{a}
MCV (Fl)	86.50±2.60^{d}	0.00±0.00	64.00±32.00^{a}	66.67±33.33^{b}	77.33±38.67^{c}
MCH (Pg)	28.50±1.44^{c}	0.00±0.00	22.00±11.00^{a}	22.00±11.00^{a}	25.33±12.67^{b}
MCHC (g/dl)	32.00±1.73^{c}	0.00±0.00	19.33±9.67^{a}	20.00±10.00^{b}	19.33±9.67^{a}
PLT (Cmm)	227.50±7.79^{c}	0.00±0.00	162.67±81.33^{b}	162.00±81.00^{b}	142.67±71.33^{a}

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤ 0.05.

Table 3.6 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd supplemented with vitamin A for a period of twelve weeks

Parameters	Treatments				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	8.00±0.00^{a}	8.67±4.33^{b}	8.00±4.00^{a}	0.00±0.00	0.00±0.00
RBC (Mil/mm^3)	3.45±0.14^{b}	1.87±0.94^{a}	2.00±1.00^{a}	0.00±0.00	0.00±0.00
Hb (g/dl)	11.50±0.29^{b}	5.80±2.91^{a}	5.93±2.97^{a}	0.00±0.00	0.00±0.00
PCV (%)	34.50±0.87^{b}	17.33±8.67^{a}	17.33±8.67^{a}	0.00±0.00	0.00±0.00
MCV (Fl)	100.50±6.64^{c}	61.33±30.67^{b}	53.33±28.67^{a}	0.00±0.00	0.00±0.00
MCH (Pg)	34.50±3.18^{b}	20.66±10.34^{a}	19.33±0.00^{a}	0.00±0.00	0.00±0.00
MCHC (g/dl)	30.00±0.00^{c}	20.66±10.34^{b}	19.33±9.67^{a}	0.00±0.00	0.00±0.00
PLT (Cmm)	247.00±5.20^{c}	147.33±73.67^{b}	142.66±71.34^{a}	0.00±0.00	0.00±0.00

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤ 0.05.

In the case of samples exposed to CdVC for a period of four weeks, there were slight increases in WBC values. Increased values of PLT were also recorded in all treatments (similar to values of CdVA treatments). After eight weeks of exposure, there were increased productions in values of WBC, lower values of RBC, Hb and PCV in all treatments. With the exception of T1 and T2, there were drastic decreases in PLT values in all treatments. Furthermore, after twelve weeks of exposure, there were increased values of WBC, decreased values of RBC, Hb, PCV, MCV and MCH; and lower PLT values in samples that survived till the end. From the statistical analysis, at week 4 of the exposure, the WBC mean values in T2 and T4 were significantly higher than T1, T3. Likewise, the T2 and T3 Hb mean values were significantly higher than T1 and T4. Meanwhile after the 8th week of exposure, the WBC, RBC, Hb, PCV, MCV, MCH and PLT mean values had no significance differences. Furthermore, after the 12th week of exposure, there were also no significant differences in the mean values of WBC, Hb, PCV, MCV, MCH and MCHC. (Tables 3.7- 3.9).

Table 3.7 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd supplemented with vitamin C for a period of four weeks

Parameters	Treatments				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	9.80±0.12^a	11.00±0.00^{ab}	11.50±0.29^b	10.50±0.29^{ab}	11.50±0.87^b
RBC (Mil/mm^3)	3.60±0.06^a	3.35±0.09^a	3.40±0.00^a	3.55±0.09^a	3.45±0.03^a
Hb (g/dl)	10.05±0.14^c	9.00±0.05^a	9.65±0.03^b	9.55±0.03^b	9.20±0.17^a
PCV (%)	29.50±0.29^c	27.00±0.00^a	28.50±0.29^b	28.00±0.00^{ab}	27.00±0.58^a
MCV (Fl)	81.50±2.02^b	80.50±2.02^{ab}	83.50±0.87^c	78.50±2.02^a	78.00±2.31^a
MCH (Pg)	27.00±0.58^{ab}	27.00±0.58^{ab}	28.00±0.00^b	26.50±0.29^{ab}	26.00±0.58^a
MCHC (g/dl)	33.50±0.29^b	35.00±0.58^c	28.50±0.29^a	29.00±0.00^a	28.00±0.58^a
PLT (Cmm)	206.00±1.15^a	239.00±12.70^d	227.00±1.73^b	227.50±6.06^b	230.00±0.00^c

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤0.05.

Table 3.8 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd supplemented with vitamin C for a period of eight weeks

Parameters	Treatments				
	CR	**T1**	**T2**	**T3**	**T4**
WBC (10^9cells/L)	9.40±0.23^b	11.50±0.87^c	6.53±3.27^a	11.00±0.00^{bc}	7.33±3.67^a
RBC (Mil/mm^3)	3.60±0.12^c	3.60±0.06^c	2.13±1.07^a	3.00±0.00^b	2.00±1.00^a
Hb (g/dl)	10.45±0.03^c	9.95±0.29^b	6.87±3.43^a	10.70±0.35^c	6.40±0.30^a
PCV (%)	31.00±0.00^{bc}	29.50±0.29^b	20.00±10.00^a	30.00±0.00^b	19.33±9.67^a
MCV (Fl)	86.50±2.60^c	82.00±2.31^b	62.00±31.00^a	105.00±2.89^d	62.00±31.00^a
MCH (Pg)	28.50±1.44^c	27.50±0.29^b	20.67±10.33^a	34.50±0.87^d	21.33±10.67^a
MCHC (g/dl)	32.00±1.73^c	29.50±0.29^b	20.00±10.00^{ab}	30.00±0.00^b	19.33±9.67^a
PLT (Cmm)	227.50±7.79^c	235.50±0.87^c	152.00±76.00^b	229.50±7.22^d	150.00±75.00^a

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤ 0.05.

Table 3.9 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd supplemented with vitamin C for a period of twelve weeks

Parameters	Treatments				
	CR	**T1**	**T2**	**T3**	**T4**
WBC (10^9cells/L)	8.00±0.00^b	6.67±3.34^a	6.67±3.34^a	0.00±0.00	0.00±0.00
RBC (Mil/mm^3)	3.45±0.15^b	2.13±0.17^a	2.00±1.01^a	0.00±0.00	0.00±0.00
Hb (g/dl)	11.50±0.29^b	6.53±3.27^a	6.53±3.27^a	0.00±0.00	0.00±0.00
PCV (%)	34.50±0.87^b	19.33±0.97^a	19.33±0.97^a	0.00±0.00	0.00±0.00
MCV (Fl)	100.50±0.67^b	64.00±32.00^a	64.00±32.00^a	0.00±0.00	0.00±0.00
MCH (Pg)	34.50±3.18^b	21.33±10.67^a	21.33±10.67^a	0.00±0.00	0.00±0.00
MCHC (g/dl)	30.00±0.00^b	20.00±10.00^a	20.00±10.00^a	0.00±0.00	0.00±0.00
PLT (Cmm)	247.00±5.20^c	158.66±79.43^b	69.33±34.67^a	0.00±0.00	0.00±0.00

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤ 0.05.

In samples exposed to CdVE treatments after four weeks, displayed higher values of WBC, slightly lower values of RBC, Hb and PCV in all treatments. There were marked increases in the production values of blood PLT in all treatments. After eight weeks of exposure, there were increased values of WBC, slightly lower values of RBC, Hb and PCV in all treatments. The increased values of MCV were recorded in T2 to T4. Moreover, after the 12th week, there were also increased values of WBC, decreased values of RBC, Hb, PCV, MCV, MCH, MCHC and PLT in the sample that survived till the end. From the statistical analysis, after 4 weeks of exposure, the WBC mean values in T2 and T4 were significantly higher than in other treatments. PCV mean values in T2 were significantly higher than other treatments. Also, the MCH mean values were significantly higher in T2. MCHC mean values in T1 were significantly higher than other treatments. After 8 weeks of exposure, the WBC mean values in T4 are significantly higher than other treatments. MCV mean values in T2 and T3 were significantly higher than other treatments. MCH mean values in T3 were significantly higher than other treatments. In addition, at the end of the 12th week, WBC mean values in T2 were significantly higher than other treatments. The RBC mean values in T1 were significantly higher than other treatments. (Tables 3.10- 3.12).

Table 3.10 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentrations of Cd supplemented with vitamin E for a period of four weeks

Parameter	Treatment				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	9.80±0.12^a	11.00±0.00^{ab}	11.50±0.29^b	10.50±0.29^{ab}	11.50±0.87^b
RBC (Mil/mm^3)	3.60±0.06^a	3.35±0.09^a	3.40±0.00^a	3.55±0.09^a	3.45±0.03^a
Hb (g/dl)	10.05±0.14^b	9.00±0.06^a	9.65±0.03^{ab}	9.55±0.03^a	9.20±0.17^a
PCV (%)	29.50±0.29^c	27.00±0.00^a	28.50±0.29^b	28.00±0.00^{ab}	27.00±0.58^a
MCV (Fl)	81.50±2.02^b	80.50±2.02^{ab}	83.50±0.87^c	78.50±2.02^a	78.00±2.31^a
MCH (Pg)	27.00±0.58^{ab}	27.00±0.58^{ab}	28.00±0.00^b	26.50±0.29^{ab}	26.00±0.58^a
MCHC (g/dl)	33.50±0.29^b	35.00±0.58^c	28.50±0.29^a	29.00±0.00^a	28.00±0.58^a
PLT (Cmm)	206.00±1.15^a	239.00±12.70^d	227.00±1.73^b	227.50±6.06^b	230.00±0.00^c

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤0.05.

Table 3.11 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentration of Cd supplemented with vitamin E for a period of eight weeks

Parameters	Treatments				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	9.40±0.23^a	14.50±0.87^c	12.50±0.29^b	13.00±0.00^b	16.00±0.00^d
RBC (Mil/mm^3)	3.60±0.12^b	3.25±0.14^{ab}	3.00±0.00^{ab}	2.85±0.03^a	2.90±0.06^a
Hb (g/dl)	10.45±0.03^c	8.85±0.26^a	9.80±0.00^b	9.60±0.12^b	8.80±0.06^a
PCV (%)	31.00±0.00^c	25.00±0.87^a	29.00±0.00^b	28.50±0.29^b	26.00±0.00^a
MCV (Fl)	86.50±2.60^b	78.50±0.87^a	96.00±0.00^d	100.00±0.00^e	89.00±1.73^c
MCH (Pg)	28.50±1.44^b	27.00±0.58^a	32.00±0.00^d	33.00±0.00^e	30.00±0.58^c
MCHC (g/dl)	32.00±1.73^b	28.50±0.29^a	29.00±0.00^a	29.00±0.00^a	29.50±0.29^{ab}
PLT (Cmm)	227.50±7.79^c	221.50±5.48^b	238.50±14.14^e	232.00±0.58^d	214.70±3.66^a

Values are presented as mean±SEM (Standard error of mean). Values with different alphabets as superscripts in a row are significantly different at P≤ 0.05.

Table 3.12 Haematological parameters of *C. gariepinus* exposed to sub-lethal concentration of Cd supplemented with vitamin E for a period of twelve weeks

Parameters	Treatments				
	CR	T1	T2	T3	T4
WBC (10^9cells/L)	8.00±0.00^a	9.33±4.67^b	15.00±0.00^c	0.00±0.00	0.00±0.00
RBC (Mil/mm^3)	3.45±0.14^c	1.87±0.93^a	2.80±0.00^b	0.00±0.00	0.00±0.00
Hb (g/dl)	11.50±0.29^c	6.33±3.17^a	7.90±0.81^b	0.00±0.00	0.00±0.00
PCV (%)	34.50±0.87^c	18.67±9.33^a	23.00±2.31^b	0.00±0.00	0.00±0.00
MCV (Fl)	100.50±0.64^c	66.67±33.33^a	82.00±8.08^b	0.00±0.00	0.00±0.00
MCH (Pg)	34.50±3.18^c	22.67±11.33^a	28.00±2.87^b	0.00±0.00	0.00±0.00
MCHC (g/dl)	30.00±0.00^c	19.33±9.667^a	29.00±0.00^b	0.00±0.00	0.00±0.00
PLT (Cmm)	247.00±5.20^c	142.67±71.33^a	213.50±0.29^b	0.00±0.00	0.00±0.00

Values are presented as mean±SEM (Standard error of mean) .Values with different alphabets as superscripts in a row are significantly different at P≤ 0.05.

3.2 DISCUSSIONS

Fish blood has two major types of cells as RBC and WBC. In fish blood erythrocytes are the most abundant cells as these contain haemoglobin that facilitates the transport of oxygen from the gills to different parts and shows pink colour when stained with Giemsa staining solution (Satish *et al*., 2018). At the initial stage (4 weeks) when samples were exposed to sub-lethal concentrations of Cd there were increased productions or generations of white blood cells (WBC) in all treatments; which further increased in both the 8th and 12th week of exposure of the samples more than the values obtained in the control. The WBC mean values in T1-T4 were significantly higher than that of the control after the 4th week of exposure. This is probably because the fishes in such unpleasant situation had to up-regulate their defence mechanism to counter the effects of the toxicant and ensure survival. The white blood cells were probably engaged in the fight against the xenobiotics in their immediate environment. This is in line with the findings of Nwali *et al*. (2018) when they reported that, white blood cell (WBC), neutrophil, lymphocyte, eosinophil, monocyte, and basophil counts, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and levels of platelets were significantly ($p<0.05$) higher in *C. gariepinus* collected from rivers close to mining sites when compared with the control.

There was a reduced production in the values of RBC, Hb, PCV and MCHC which were concentration and duration dependent. The blood platelets also towed the same line and became more drastically reduced in the 8th and 12th weeks of exposure. This is probably due to their usage in the presence of the toxicant to combat the oxidative stress elicited by it. Reduction in cellular blood iron resulting in the reduced oxygen carrying capacity of blood and ultimately stimulating erythropoiesis can probably determine the amount of RBC availability with increasing concentration of toxicant. Adebayo and Fapohunda (2016) demonstrated how haematological changes reflected a highly significant reduction in Red Blood Cell counts from T 1 (3.78+ 0.46/µl) to T4 (2.68 + 0.18/µl); and also recorded a significant decrease in haemoglobin (Hb) from 12.25±0.5g/L in control (0ml/70L of water) to 7.83±0.99g/L in T4 (65ml/70L). This is also in conformity with the findings of Khan *et al*. (2019) when they reported a significant decline in mean haemoglobin, corpuscular haemoglobin and packed cell volume in treatments exposed to Cd and Hg. Other findings have also linked the reduction of these parameters to the presence of one toxicant or the other. For instance, anomalies in blood cell parameters including cell membrane damage and nucleus shrinkage in RBCs confirmed the toxicity of $BaCO_3$ on *Channa punctatus* (Zorawar *et al*., 2017); and exposure of *platichthys stellatus* to varying concentrations of chromium led to decreased hepatosomatic index, RBC, Ht and Hb decreased significantly after exposure to 400ppb for 2 weeks (Ko *et al*., 2019).

In samples exposed to CdVA treatments there was an increased generation of WBC and PLT in all treatments in the first 4 weeks of exposure. The increases in WBC continued in the 8th and 12th weeks of exposure with higher significance difference in T1-T4. There was probably a constant need for up-regulating of the defence mechanisms in order to maintain a constant physiological balance that ensured the tolerance of the organism to the presence of the xenobiotic. This is probably because haematological parameters have been considered good indicators of the physiological changes and health status in fish (Burgos-Aceves *et al*., 2019). And these parameters can be altered by metal exposure (Fazio *et al*., 2014). Also, the presence of vitamin A was probably not felt in attenuating the effects posed by the xenobiotic. MCV and MCHC values were also increased during the 4th week of exposure. However, at the 12th week of exposure the RBC, Hb, PCV, MCV, MCH, MCHC and PLT values were all reduced. It was also evident that, the higher the concentration of the toxicant the lower the production level or amount of the PCV after the 4th week of exposure. At the 12th week there was no significant difference in the production levels of all the parameters. Similar findings were reported by Kaoud *et al*. (2011) on exposure of Nile Tilapia to Cd which resulted in a significant reduction of erythrocyte count (RBC), haemoglobin content (Hb) and haematocrit value (Hct). A significant decrease in RBC, Hb, PCV, MCHC and MCH was also reported in fish exposed to different concentrations of ambient inorganic mercury (Pratap, 2016). Growth and hematological parameters measured decreased with increasing arsenic concentration, while the concentration of plasma components measured increased (Han *et al*., 2019). The results obtained indicated significant ($P<0.05$) reductions with increased concentrations of the chemical in haemoglobin (Hb), Red blood Cell (RBC), packed cell volume (PCV), lymphocytes, platelets and mean corpuscular volume (MCV). The similar findings indicated that the white blood cell (WBC), neutrophils, monocytes, mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentrations (MCHC) in fish exposed to the pesticides were significantly ($P<0.05$) higher than that of the control (George *et al*., 2017).

From the results of the analysis of the samples exposed to sub-lethal concentrations of Cd and supplemented with vitamin C there was a slight increase in the values of WBC and PLT in all the treatments after 4

weeks. This is probably because of the presence of vitamin C such that immediate mass production of body defence mechanisms in the form of white blood cells was not necessitated. There were however, increased levels of production of WBC in the 8th and 12th weeks of exposure. There were also reduced values of RBC, Hb, PCV, MCV and PLT (more drastic in T3 and T4). The RBC, PLT, MCHC and Hb mean values were significantly higher than in T1-T4. At these stages of exposure the body's defence mechanisms may have been overwhelmed; hence, the need for up-regulation of the physiological status of the fish to ensure survival by increasing the production of WBC and utilization of RBC (and other parameters) in order to upset oxygen deficit occasioned by the presence of the toxicant especially at higher concentrations. Bell *et al*. (2013) posited that reduction in haem synthesis occurs due to the effects of pollutants that lead to inhibition of RBCs and reductions in haemoglobin levels due to the presence of the pollutants. These findings from this research are in conformity with Khan *et al.* (2019) when they reported a significant decline in mean haemoglobin, corpuscular haemoglobin and packed cell volume in treatments exposed to Cd and Hg; and that chemo-treatment with vitamin C reduced the effects of Cd and Hg, and their co-administration indicative of the probable shielding effects of the vitamin C on metal toxicity. In like manner, Maurya *et al*. (2019) reported elevated levels of WBCs and decreases in RBCs, MCH, haemoglobin levels, PCV, MCV (except in 10% treatment). In addition to these, studies have shown that antioxidative vitamins such as C and E, which are widespread in many food products, have been shown to have a mitigation effect in heavy metals toxicity (Mehrpak *et al*., 2015; Asaikkuttia *et al*., 2016 and Sahiti *et al.*, 2018).

In samples exposed to sub-lethal concentrations of Cd and supplemented with vitamin E, there was increased production of WBC, reduced RBC, Hb, PCV and marked increases in PLT levels in all treatments after the 4th week of exposure. As the duration and concentration increased there were increased WBC, reduced RBC, Hb, PCV, and increased MCV in T2-T4, MCH, MCHC and PLT after 8th and 12th weeks of exposure. The WBCs were also significantly higher in T2 and T4 at week 8 and significantly higher in T2 only at week 12. T2 seems to be the optimal elicitation point of the toxicant. The body's defence mechanisms were probably up-regulated from the beginning to deal with the deleterious effects of the toxicant. The red bood cells and other parameters were also utilized in the defence of the system. The marked or elevated production of the platelets at the initial stage may have been occasioned by the need for urgent repair and clotting of the injury or damage inflicted on the fish by the toxicant in order to restore physiological balance and ensure survival since, haematology is an indicator of immunological status and can provide a definitive diagnosis of fish during toxicant exposure (Nte *et al*., 2011). Also in line with the findings of this research, Madhusudan *et al*. (2015) reported that an increased level of platelets was a response to the need to repair damaged organs by the toxicant and increased values of WBC could be due to generalized immune response and protective response to the toxicant stress. In addition to this, Tezcan *et al*. (2012) have reported that vitamin E and vitamin C together may prevent cytotoxic damage of erythrocytes at low and moderate Cd concentrations; and that the changes in haematological, biochemical and antioxidant parameters were restored in the fish fed with vitamin E supplemented feeds (Azeez and Braimah, 2020). Likewise, Vitamin E reversed the anemia triggered by a decrease in erythrocyte count, haematocrit and haemoglobin level of lead-exposed *Rattus norvegicus*, and that administration of both vitamins E and C together proved more efficient than either of the two singly (Xhyrel *et al*., 2016). Furthermore, a similar finding was reported by Gupta *et al*. (2013) when they recorded a significant decrease in RBC, MCHC, Hb and PCV, an increase in WBC, MCV and MCH values of minor carp, *P. sophore* exposed to $CuS0_4$.

CONCLUSIONS AND RECOMMENDATION

The samples of *C. gariepinus* exposed to sub-lethal concentrations of $CdCl_2$ displayed varying levels of amelioration of the effects elicited by the presence of the xenobiotic with increased values of production of WBC and PLT in all treatments with increasing duration of exposure. The effects of the vitamins were evident in lower concentrations than in higher concentrations.

In the case of samples exposed to CdVC there were slight increases in WBC and PLT values in all treatments. Samples exposed to CdVE treatments after four weeks, displayed higher values of WBC, and marked increases in the production values of blood PLT in all treatments. The RBC and associated parameters also decreased significantly especially in higher concentrations in the presence of the vitamins. The vitamins supplemented treatments displayed varying levels of ameliorations far better than the Cd only group. Amongst these, the CdVC and CdVE treatment groups fared better than others.

The outcome of this research has shown the impacts of vitamins A, C and E in mitigating the effects of the toxicant and can serve as a remedy in heavy metal toxication when appropriate concentrations are administered.

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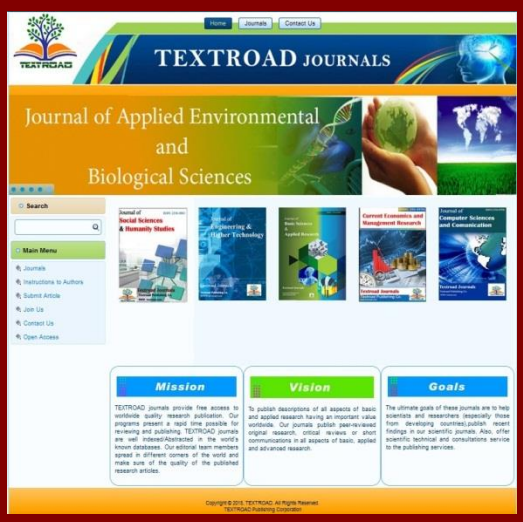

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