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Research Article

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Hematological and Biochemical Investigations of Green Synthesized Reduced Graphene Oxide Carbon Nanoparticles on Acetaminophen Induced Male Albino Rats.

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Abstract: The aim of this study is to evaluate the protective activity of green-synthesized Reduced graphene oxide carbon nanoparticles (RGO-CNP) from *Asparagus racemosus* (shatamuli) by hematological and biochemical assessments on acetaminophen induced uremic rats. 36 animals were divided into six groups including a control group, acetaminophen induced uremic group and nanoparticle treatment groups. Nanoparticle treated four groups were intraperitonealyco-administered RGO-CNP at doses of 0.5, 1.0, 2.0 and 4.0 mg/kg bw and acetaminophen at 500 mg/kg ofbw for 14 days respectively. Hematological analysis revealed that there was significant decrease ($p<0.05$) in Red Blood Cells (RBCs), Hemoglobin (Hb), with acetaminophen induced Group II. Co-administration of green synthesized RGO and acetaminophen on nanoparticle treating groups showed the effective protectivity and significant ($p<0.05$) recovery in the hematological alterations. Other hematological parameters including- WBC, Platelet, Hematocrit, MCH and MCHC remained within the reference range and no significant alterations were observed. There was a significant increase in plasma ALT, AST, serum urea and creatinine levels ($p< 0.05$) in uremic group (Group II) compare to control (Group I).The alterations were further resettled to the higher dose of RGO treated groups (Group V and VI). The other tested biochemical parameters ALP, total bilirubin, direct bilirubin, Serum triglyceride and cholesterol levels were not significantly altered when compared with control Group I. In conclusion, it is established that RGO possess protective response against acetaminophen induced toxicity as dose responsive manners, which is supported by haematological and biochemical observations. This promising effect suggests the potential applicability of RGO as therapeutics in drug delivery systems to reduce the toxicity.

Keyword: Acetaminophen, Biochemical assay, Carbon nanoparticles, Hematologicalstudy ,Reducedgraphene oxide, Uremia.

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1. INTRODUCTION

During the past few years, graphene-based nanomaterials (GBN) have been attracted researchers to its use in wide range of biomedical applications including biosensors, photothermal therapies, tissue engineering and regenerative medicine not only because of their outstanding physical, chemical and biological properties, but also the specific arrangements of grapheme which provide a robust absorbing capability and makes it a novel carrier for drug delivery[1]. Graphene pharmacology and *in vivo* studies are still in infancy, with the need for expansion. Although a number of scientific investigations reported the cytotoxic behaviour of GBN, but some others findings suggest that future prospects of GBN are very bright and optimistic. Combination of GBNs with other material compositions that have prior flexibility or functionality makes them better candidates for fabrication of multifunctional smart materials. The investigations of *Kurantowicz et al*.[2] shows that tested Carbon nanoparticles(CNP) or GBN had no toxic effects on general animal health status, growth, overall appearance of the animal interior, organ weight, and biochemical and hematological parameters. The particles accumulated as small dots (<1 μm in diameter) and massive agglomerates in proximity to the injection sites. The tendency of CNP to form agglomerates is unique and could be useful in drug delivery systems to immobilize CNP and drug complexes in the targeted body regions and then slowly release active substances. According to Mendonça*et al*. (2016) systemic administration of reduced graphene oxide leads to minor toxic effects in blood, but offers no significant health risk in experimental rat model [3]. In a separate study considerable emphasis has been given to the safety assessment of reduced graphene oxide nanoparticles. From all the toxicity endpoints studied in human PBMCs, they concluded that the tested nanoparticle could be considered biologically safe at concentrations below100 μg/ml. [4]*In vivo* drug delivery is an expanding field involving loading of graphene materials with therapeutic agents due to the high surface area such as the anticancer drugs doxorubicin and curcumine[5]. Due to the high flexibility of GBNs reduced graphene oxide makes scaffolds during its reduction by reducing substances, which is very useful for superimposing soft biological systems and integrating within cellular environments[6-7]. The differences in the preparation methods and physicochemical characteristics of these materials result in dramatically different biological behaviour *in vivo*. Synthesis of reduced graphene oxide by using phytochemicals from various medicinal plants could be utilized in the therapeutic approach of crucial disorders management. This ecofriendly and easily available green synthesis technique is not only cost effective but also performable as a superior drug vehicles by holding the functional compounds in its scaffoldings core. The objective of this study was to determine the effect of green synthesized reduced graphene oxide carbon nanoparticles on haematological and biochemical blood markers on acetaminophen induced albino rats.

Figure 1: Photograph of Asparagus racemosus (Shatamuli)

2. MATERIALS AND METHODS

2.1 Reagents

Acetaminophen (paracetamol, *N*-acetyl *p*-aminophenol; APAP) was purchased from AshChemie India. It was administered intraperitoneally with saline water. Graphene oxide was obtaining from our collaborative laboratory; Dept. of Chemistry, IIEST, Shibpur, West Bengal, India. Diagnostic kits for serum urea, creatinine, ALP, SGOT, SGPT, Total bilirubin, Direct bilirubin, Triglyceride and Cholesterol assays were obtained from Agappe Diagnostics Ltd. India. Other chemicals and reagents used in this study were of analytical grade.

2.2 Preparation of nanoparticles

In our laboratory we have prepared reducedgraphene oxide carbon nanoparticles from graphene oxide by using modern green synthetic approach[29,30]. Root extracts of *Asparagus racemosus* (commonly known as Shatamuli) (Figure 1) has been used as reductant phytochemicals for this green conversion. Characterization of the prepared nanoparticle was done by – UV visible spectroscopy, Transmission Electron Microscopy (TEM), EDX, XRD, FTIR, DLS and Zeta Potential measurements. Optimized results from that study were accepted in scientific journal and pursuing for publication at press (Figure 2). In this investigation research work the same above mentioned nanomaterials were used.

2.3 Toxicity study: Animal grouping, maintenance and treatments

Thirty-six healthy adult male Wister strain rats with average body weight of 125 ± 5 g were purchased from authorized Chakraborty Animal suppliers, Kolkata (M/S ChakrabortyEnterprise Registration no.: 1443/PO/b/11/CPCSEA) and used in this study. The animals were maintained under standard environmental setup and acclimatized in the laboratory conditions for a week beforethe onset of the study. In this period, they were provided normal diet with complete balanced nutritional value suitable for biomedical research. Rats were divided into six groups with each group containing six rats (n=6). Animals of Group I were served as control group, subjected to feed dry food (pellet diet) and an adequate amount of water. They received de-ionized water for 7 days prior to experimentation, followed by the next 14 days of experimental period. Group II, Group III, Group IV, Group V and Group VI animals were given acetaminophen at the dose of 500 mg/ kg of body weight with de-ionized water per day for 10 days intraperitoneally to achieve uremia. The selective dose of acetaminophen induced uremic development was reported and already established in our laboratory[8]. Group II animals were then treated as uremic group. Animals of Group III, IV, V, and VI were co-administered the selective nanoparticles by using aqueous solvent intraperitoneally at 0.5, 1.0, 2.0 and 4.0 mg/kg of body weight (according to the OECD guidelines, 2000) [9] respectively for 14 days. All the animals of treated groups provided normal diet with adequate water in this experimental period. Experiments were performed and complied as per the rules of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (Registration No: 1905/PO/Re/S/2016/CPCSEA) and the study was approved by the Institutional Animal Ethical Committee (IAEC) (Ref. No. 04/IAEC (3)/S/RNLKWC/2017) of the Raja N. L. Khan Women's College, West Bengal, India.

2.4 Hematological assay

After completion of treatment, the rats were fasted for overnight; then anesthetized with ether inhalation. Blood samples were collected by cardiac puncture into EDTA vacuum tubes for haematological study and into sterile centrifuge tube for biochemical assay. Erythrocytes count, Hemoglobin (Hb) level,haematocritvalue,Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC),total leukocytes and platelets count were determined by automatic whole blood analyzer. The other blood samples were left for 1 h at room temperature then centrifuged at 2,000g for 3 min to get serum. Then, the serum was collected and stored at -20ºC for subsequent biochemical assays.

2.5 Biochemical assay

The biochemistry of the hemolysis free serum was analyzed by commercially available standard kit by using a semi-autoanalyzer (AGAPPE Diagnostic Ltd., India) according to the manufacturer's instructions and maintaining standard protocol. The following biochemical parameters were analyzed: serum urea, serum creatinine, ALP, SGOT, SGPT, Total bilirubin, Direct bilirubin, Triglyceride and Cholesterol.

2.6 *Statistical analysis*

All results are expressed as means ± SE. Serial measurements were analyzed by using one-way ANOVA with Tukey's t test, Bonferroni test using Origin 6.1 (Northampton, MA, USA) software. The critical significance level α was 0.050 and, then, statistical significance was defined as $p<0.05$.

3. RESULTS

3.1 Hematological study

In this study it has been observed that RBCs count and Hb level are significantly decreased ($p < 0.05$) after intraperitoneal administration of Acetaminophen on Group II animals compared to Control (Group I). Further in contrast with Group II, co-administration of acetaminophen with green synthesized reduced graphene oxide (RGO) carbon nanoparticles on different doses (0.5, 1.0, 2.0, 4.0 mg/kg BW) RBCs count and Hb level are increased progressively. Although, close similarities were observed among Group III, IV and Group II; but Group V and VI were profoundly rises these haematological values and significantly differed ($p<0.05$) from Group II. Other hematological parameters like – WBC, Platelet, Hematocrit, MCH and MCHC remained within the reference range. There are no significant changes observed in these hematological parameters in comparison with the control (Group I), acetaminophen induced uremic (Group II) and nanoparticles co-administered intra-group (Group III to VI) analysis.

3.2 Hepatotoxicity Study

The elevated levels of ALT, AST and ALP are indicative of cellular leakage and loss of functional integrity of the hepatic cell membranes implying hepatocellular damage.[10] Results from that study showed that there is a marked increase ($p< 0.05$) in the levels of serum liver enzymes – AST and ALT in rats treated with acetaminophen alone in Group II. However, serum ALP level was also increased in Group II, but this was statistically not significant when compared with control Group I. On the other hand, animals co-administered with RGO with acetaminophen decreases the AST and ALT level slowly at group III with the progressive increasing of RGO doses, although on Group III and IV the enzymes levels are still higher and significant changes ($p< 0.05$) observed with control Group-I. But in the group V and VI animals there was no significant alterations were found when compared to control group. The ALP levels are not significantly altered by co-administered groups (Group III to VI), when compared with control group and by intra-group analysis (Table 1). There were no harmful changes found in direct bilirubin and total bilirubin levels between acetaminophen induced group II and Acetaminophen-RGO co-administered groups (III to VI) when compared with control group I. The direct bilirubin and total bilirubin concentrations of all tested animals remained within the reference range. [11]

3.3 Reno-protective Study

In this study, for the assessment of renal functionality serum urea and creatinine levels of the tested animals were measured by specified kit. There was a significant ($p<0.05$) increase in the plasma urea and creatinine concentrations in the Group II animals were found in comparison with Group- I. The serum

creatinine levels of the control group were 1.005 mg/dl which remained in the normal range of serum creatinine in male rat (0.3–1.2 mg/dl) [12]. The elevated creatinine concentration indicates the achievement of uraemia in acetaminophen induced Group II animals.[13] In Group III and IV, the creatinine levels are markedly found and significantly differ from control group (P<0.05). As dose dependent manner Group V and VI animals co-administered with acetaminophen and relative higher dose of RGO, the serum creatinine levels are decreased and no significant alterations were observed when compared with control group (Figure 4). In the same manner, serum urea concentrations are also measured by semi-automated analyser using specific kit. Serum urea concentrations present at an average level of 26.52 mg/dl in control group of animals, where as it was significantly increased (P<0.05) to 56.81 mg/dl in acetaminophen induced uremic Group II. In the Group III, IV, V and VI the concentrations are 49.95, 50.35, 39.02 and 37.08 mg/dl respectively (Figure 3).

3.4 Lipid Assay

Lipid profile measurements did not show a statistically significant difference between the intra group analysis. Figure 5 and 6 represents the experimental data obtained from the analysis of serum cholesterol and triglyceride concentrations.

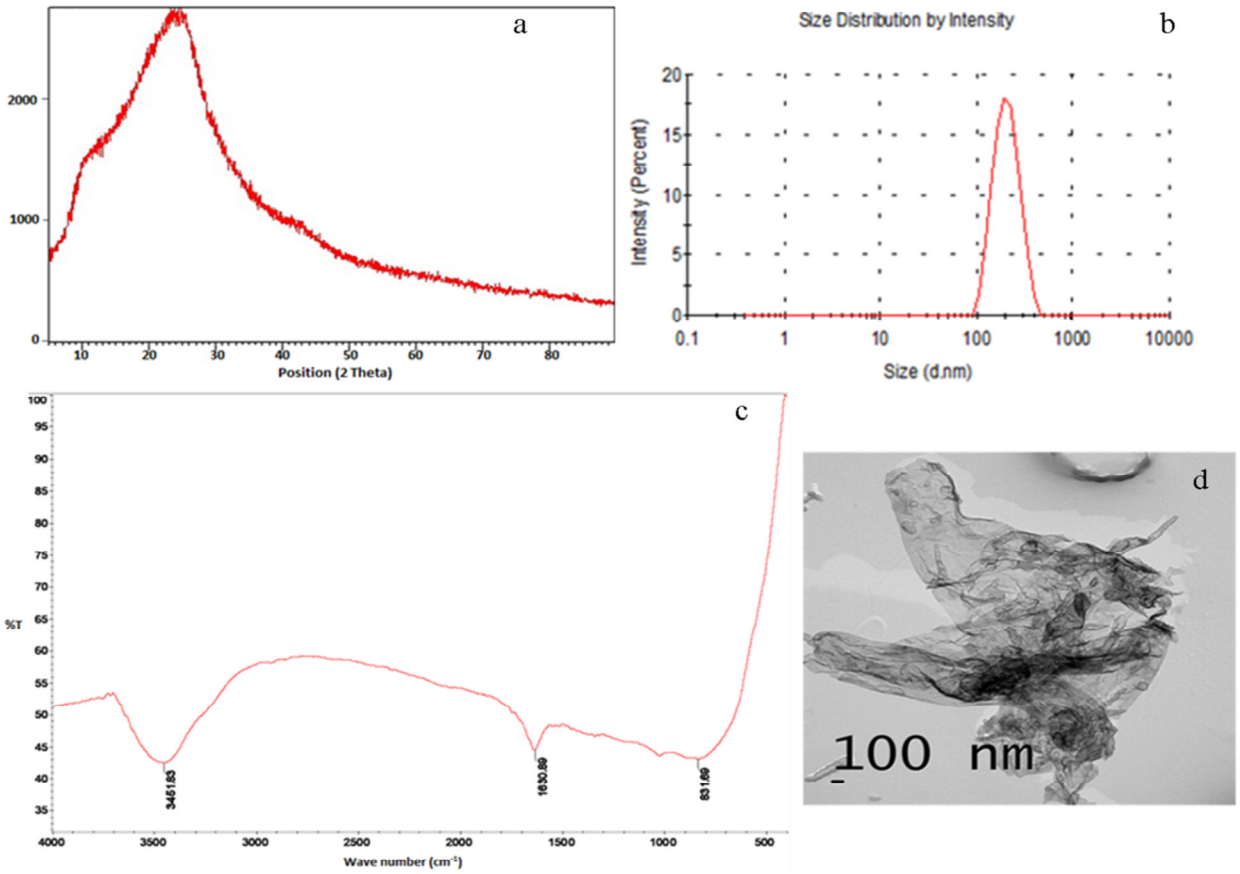

Figure 2 Characterization of RGO: Different characterizing features of studied RGO – (a) XRD spectrum of RGO (b) DLS pattern of RGO (c) FTIR study of RGO, (d)) HRTEM image.

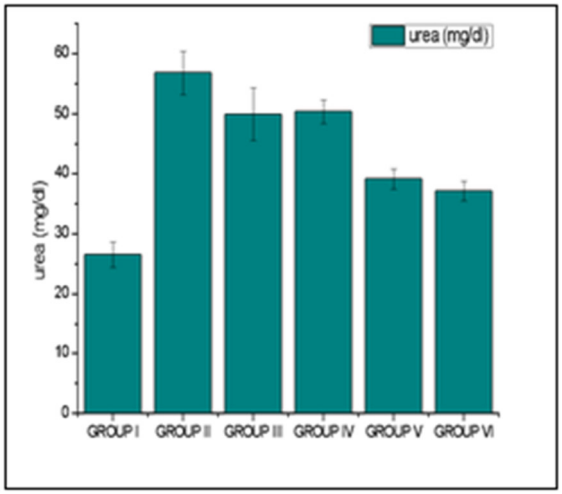

Figure 3 Effect of biogenic RGO nanoparticles on serum urea level on acetaminophen induced hepato-renal toxicity of rats.
Data are expressed as Mean ± SE (n=6). One-way ANOVA followed by Tukey test, Bonferroni post hoc test (p<0.05).

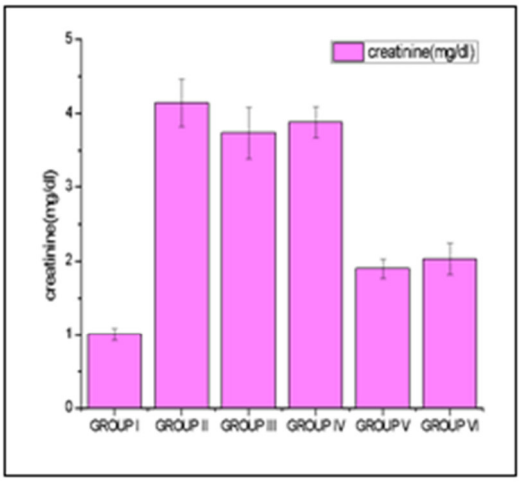

Figure 4 Effect of biogenic RGO nanoparticles on serum creatinine level on acetaminophen induced hepato-renal toxicity of rats. Data are expressed as Mean ± SE (n=6). One-way ANOVA followed by Tukey test, Bonferroni post hoc test (p<0.05). Legend

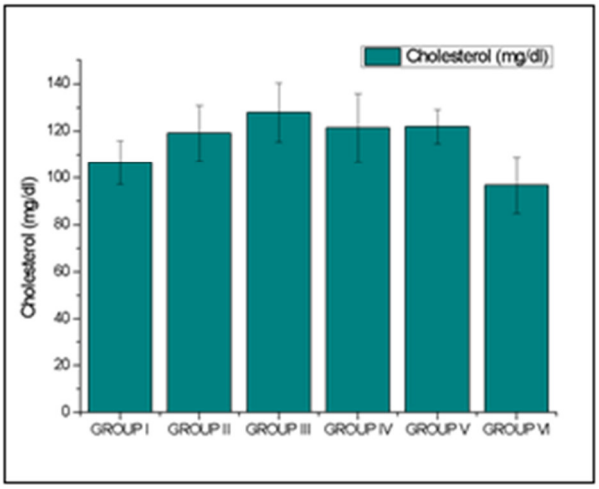

Figure 5 Effect of biogenic RGO nanoparticles on serum cholesterol level on acetaminophen induced hepato-renal toxicity of rats. Data are expressed as Mean ± SE (n=6). One-way ANOVA followed by Tukey test, Bonferroni post hoc test (p<0.05). Legend

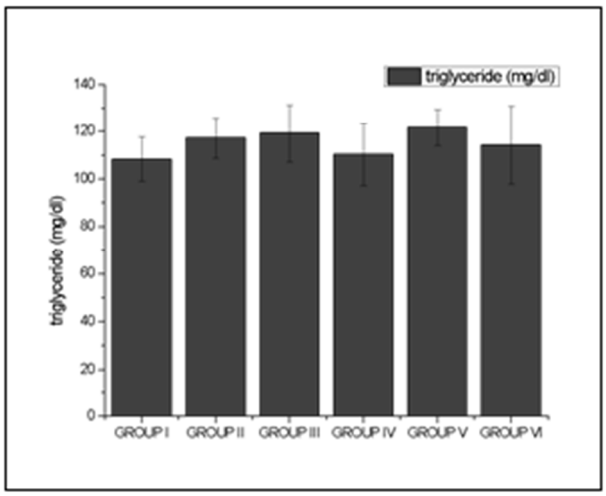

Figure 6 Effect of biogenic RGO nanoparticles on serum triglyceride level on acetaminophen induced hepato-renal toxicity of rats. Data are expressed as Mean ± SE (n=6). One-way ANOVA, followed by Tukey test, Bonferroni post hoc test (p<0.05). Legend

Table 1: Effect of RGO-CNP on the hematological parameters in blood of control and experimental animals.

Parameters	Control *Group I*	Uremic *Group II*	Uremic + RGO(0.5 mg/kg BW) *Group III*	Uremic + RGO(1.0 mg/kg BW) *Group IV*	Uremic + RGO(2.0 mg/kg BW) *Group V*	Uremic + RGO(4.0 mg/ kg BW) *Group VI*
Hb (g/dl)	12.60±0.35	9.49±0.19#	9.91±0.35#	10.68±0.26#	11.55±0.31	11.61±0.21
RBC (10^{12}/L)	8.61±0.39	7.11±0.27#	7.51±0.29#	7.29±0.30#	8.11±0.31	8.05±0.42
WBC (10^3/mm^3)	7.42±0.83	6.93±0.78	8.76±0.36	7.76±0.78	7.15±0.47	6.85±0.21
PLT (10^9/L)	910.66±52.36	838.66±78.14	908.83±68.28	885.33±53.26	844.5±64.14	734.66±74.09
Hct (%)	41.68±0.54	38.58±0.78	39.24±0.34	39.38±0.62	39.46±0.62	39.81±0.28
MCH (pg)	15.99±0.80	14.73±0.26	15.33±0.27	16.50±0.68	15.73±0.78	15.68±0.39
MCHC (g/dl)	30.25±0.95	29.54±0.63	29.40±0.65	30.44±0.52	30.24±0.81	30.43±0.54

One-way ANOVA, Tukey test, Bonferroni post hoc test; data expressed as mean ± SE, n = 6 in each group. p< 0 .05 Indication of superscripts: # = significant (p<0.05) increase compared to control. Hbhemoglobin; RBC red blood cell; WBC white blood cell; PLT platelet; Hcthematocrit; MCH mean corpuscular hemoglobin; MCHC mean corpuscular haemoglobin concentration.

Table 2: Effect of RGO on liver enzymes – ALP, AST and total bilirubin and direct bilirubin concentration.

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
ALP	247±24.33	279.61±19.54	246±27.37	250.62±29.51	242.14±14.05	232.87±15.75
AST	240.08±19.11	381.51±10.47#	326.75±14.18#	335.81±20.51#	283.70±10.65	242.66±16.59
ALT	144.74±14.29	259.66±10.58#	254.29±11.86#	239.16±22.35#	233.45±18.55	187.22±11.28
Total Bilirubin	0.28±0.01	0.32±0.07	0.44±0.09	0.33±0.06	0.27±0.01	0.42±0.08
Direct Bilirubin	0.14±0.01	0.18±0.04	0.24±0.05	0.20±0.04	0.15±0.01	0.25±0.05

One-way ANOVA, Tukey test, Bonferroni post hoc test; data were shown as mean ± SE, n = 6 in each group. p< 0 .05. Indication of superscript: (#) significant (p<0.05) increase compared to control.

4. DISCUSSION

Studies on the safety assessment of RGO invivo animal models are still not to the optimum. In this regard, there is an urgent need for more animal studies to clarify the systemic behaviours of these nanomaterials and their potential toxicities. The present study was conducted to evaluate the effect of green synthesized RGO against acetaminophen induced haematological and biochemical alterations in male albino rats. Acetaminophen chemically named as N- acetyl p-aminophenol is a widely used analgesic and antipyretic drug which is safe in therapeutic doses. [14-15] But administration of acetaminophen at overdoses can cause significant tissue damage, associated with glutathione depletion and lipid peroxidation. It results an intracellular accumulation and high reactive metabolite binding N-acetyl-p-benzoquinoneimine, liver cell damage, and often end with death. Similar effects also occur in kidney tissues[16-17]. In this present research work, we have employed a dose dependent approach of green synthesized RGO to evaluate the potential effect of this newly

developed nanomaterials against acetaminophen induced hepato-renal toxicity by haematological and biochemical assessment. In order to retain the engineered functions invivo, nanomaterials used for biomedical applications should be compatible with blood. It has been demonstrated that reduced graphene oxide (RGO) carbon nanoparticles were compatible with blood and neither cause hemolysis, platelet activation nor changes in coagulation or abnormalities in hematological parameters. In this study it was observed that RBC count and Hb concentrations are significantly decreased ($p< 0.05$) in acetaminophen induced uremic group II from control group I. Research findings from Mitra M et.al (2019) and Oyedeji K.O et.al (2013) suggest that treatment of rats with acetaminophen caused significant decrease in RBC count, which could indicate that there were destruction of matured RBC and reduction in the rate of erythropoiesis. This could also imply that acetaminophen have the potency to inhibit the secretion of erythropoietin from kidneys [18-19]. The significant decrease in Hb concentration on the acetaminophen induced group is due to destruction of RBC and as a result of reducing erythropoiesis. Similar report was given by Adedapo et al., (2007) in rats treated with *A. cordifolia* and *S. Virosa* extracts[20]. On the other side co-administration of RGO with acetaminophen in group III, IV, V and VI shows the alterations in RBC count and Hb concentration. Though group III and IV do not show the statistical significant alteration when compare with group II, but elevated blood parameters in group V and VI signify the similarities with control group I ($p<0.05$). In this contrast it is assumed that the green compound which is present in the RGO structure not only have an anti-hemolytic activity but also can play a protective effort against the acetaminophen induced haematological alterations. Phytochemicals present in the root extracts of *Asparagus racemosus* can increase the RBC count and Hb concentration, which is already established from several research findings [21-22]. AST (SGOT) and ALT (SGPT) are the enzymes normally present in the liver, heart, muscles and blood cells. They are principally located within hepatocytes. Whe.liver cells are injured or damaged, transaminases are released into blood stream,So, altered enzymes activities are the index of liver injury [23]. The effect of acetaminophen and RGO administration at different doses on biomarker enzymes for liver function was shown in table 2. The levels of AST and ALT were markedly elevated in the acetaminophen treated group compared to control. Toxic metabolites from acetaminophen, particularly N-acetyl-p-benzoquinoneimine rapidly conjugated to reduced glutathione, which is further accumulates and binds to intracellular macromolecules that can lead to cell injury, usually through apoptotic pathways. Association of acetaminophen with ALT and AST elevation as a result of hepatic toxicity is well studied [24].Administration of RGO at the relatively higher doses (group V and VI) to acetaminophen induced hepatotoxic rats restored the level of ALT and AST offering the maximum hepatoprotection with respect to different liver marker enzymes. The ALP levels, Total bilirubin and direct bilirubin concentrations are not markedly change among the tested group of animals. The similar research findings were reported by Mendonça et al (2016). The present results are in agreement with the study of Amrollahi-Sharifabadi et al (2018) and Kurantowicz et al (2015) stated that the tested RGO nanoparticles had no toxic effects on liver function associated biochemical parameters [25]. Serum urea and creatinine concentrations are the strong biomarker of renal functions, which is essentially elevated ($p<0.05$) by administrations of acetaminophen in group II from the normal level of control group I. An increased serum urea and creatinine concentration indicates renal dysfunction. In uremia it could be occur due to the accumulation of urea in the blood. The exceed production rate of plasma urea than the rate of clearance is responsible for the uremia, which leads to kidney damage [26-27]. Because of the renal dysfunction, creatinine which is mostly derived from endogenous sources is not efficiently filtrated by kidney. The elevated level of serum urea and creatinine were significantly ($p<0.05$) reversed by the higher doses of RGO (Figure 3 and 4) in group V and VI. Although in RGO treated (lower doses) group III and IV, the levels are reduced but it does not possess statistical significant status when compared with control group I. The novel compounds present in the RGO structure may be responsible for the reduction of the acetaminophen induced renal toxicity. Phytochemicals obtained from root extracts of *Asparagus racemosus*, was used for the synthesis of RGO probably have the capability to boost up the endogenous antioxidant system in the liver and kidney of rats. It could have also enhanced excretion of NAPQI and free radicals in both liver and kidneys thereby reducing toxicity of acetaminophen [28]. Effects of RGO nanoparticle on triglyceride and cholesterol level of the all tested groups were measured (Figure 5 and 6). Results are within normal reference range and no significant differences were found between intra group analysis.

5. CONCLUSION

In conclusion, the obtained findings clearly suggest that green synthesized RGO have the potential capability to protect against hepato-renal damage due to acetaminophen induced toxicity. From the toxicity endpoints study on haematological and biochemical parameters, this newly synthesized nanoparticle could be considered or recommended as biologically safe and showed not only healthy compatibility with blood, but also showed potentiality against acetaminophen induced hepato-renal toxicity. However, it represents preliminary information about the RGO, but further research is required to explore the interaction mechanism in biological system and to safely use for biomedical applications.

6. ACKNOWLEDGMENT

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7. AUTHORS CONTRIBUTION STATEMENT

The concept was prepared by Dr. DK. Nandi and Prof. C. Ghosh. Laboratory work, data collection, numerical calculation and analysis of obtained results were done by S. Paul, N. Yasmin and M. Mitra. All authors discussed the methodology and results and contributed to the final manuscript.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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