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Protective Role of Green Synthesized Gold Nanoparticles Using *Terminalia arjuna* against Acetaminophen Induced Hematological Alterations in Male Wistar Rats

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Abstract

Background: The present study aim to investigate on the characterization of green synthesized gold nanoparticles (AuNPs) and to evaluate whether this herbal nanoparticle can increase the efficiency of herb for alteration of hematological indices against acetaminophen induced toxicity in male Wistar rats.

Methods: Bark extract of *Terminalia arjuna* was used for the green synthesis of AuNPs and then characterization of the nanoparticles were done. Then experiment was conducted on 24 healthy male Wistar rats. The animals were divided into four groups, each group having six rats. Group-1: Control; Group- 2: acetaminophen treated (500 mg / kg) for 14 days; Group-3: Co-administration of acetaminophen (500 mg/kg/day) along with *Terminalia arjuna* bark extract (175 µg/kg/day) for 14 days; Group-4: Co-administration acetaminophen (500 mg/kg/day) along with of green synthesised AuNPs (175 µg/kg/day) for 14 days. Hematological indices were measured using standard hematological techniques.

Results: The green synthesized AuNPs were characterized by UV-visible spectroscopy, FESEM, HRTEM, EDX, FTIR, XRD, DLS analysis. UV-visible spectroscopy showed SPR band at 524 nm. FESEM, HRTEM and XRD analyses revealed that green synthesized AuNPs were spherical shaped, crystalline in nature with size ranging between 20 and 40 nm. Hematological analysis revealed that there was significant decrease in Red Blood Cells (RBCs), Hemoglobin (HB), Hematocrit (HCT)%, Lymphocyte percentage and Platelet Distribution Width (PDW)%, with acetaminophen treatment but White Blood Cells(WBCs), Red blood cell Distribution Width (RDW)% and Platelets (PLTs) significantly increases with acetaminophen administration. Then after co-administration with green synthesized AuNPs along with acetaminophen showed effective significant recovery in the hematological alterations.

Conclusions: Overall the results highlighted the promising effect of green synthesized AuNPs against acetaminophen induced hematological alterations in male Wistar rats.

Keywords: *Terminalia arjuna*; Gold nanoparticles; FESEM; HRTEM; Hematological indices

Introduction

Development in the field of nanotechnology has embossed the necessity of utilizing therapeutic nanoparticles for the detection and treatment of diseases. Among the metallic nanoparticles gold nanoparticles (AuNPs) has great importance because of its wider applications in drug delivery [1], biomedical [2], biosensor [3], anticancer [4], antioxidant [5] due to its biocompatibility well defined size, shape, stability and can be easily synthesized [6]. Chemical synthesis method of AuNPs is hazardous to the environment toxic to the biological system. Green synthesis of nanoparticles by using plants and its extract have received much interest due to its eco-friendliness [7,8], less biohazardous, non-toxicity, cost effectiveness and easily scalable [9]. From different studies it has been reported that flavones, polyols, terpenoids, polysaccharides and proteins are involved in the bioreduction and stabilization of the metal ions during nanoparticles synthesis using plant [10]. In last few years, for the development of nanotechnology based drugs many pharmaceutical companies have got approval from the US Food and Drug Administration(FDA) as there is a great urge for large investment in developing new nanotechnology based medical tools for therapeutics [11].

Investigations in the area of green synthesis of gold nanoparticles using living plants [12] were first reported by Gardea–Torresdey and his co-workers. Scientific research reports demonstrated that several plants were used for biosynthesis of nanoparticles, which includes *Sida acuta* leaf extract [13], *Beta vulgaris* [14], crude extract of *Syzygium aromaticum* [15], *Piper nigrum* [16]. Synthesis of AuNPs using several plants have been reported which includes *Terminalia arjuna* [17], *Morinda citrifolia L.* [18], *Murraya koenigii* [19], *Terminalia chebula* [20], *R. tuberosa*& *P. acidus* [21], and *Gnidia glauca* [22]. From environmental issues it is clear that the green synthesis meets the significant potential in using of safe, harmless, renewable materials for nanoparticle synthesis. In this current study bark extract of *Terminalia arjuna* is used for the green synthesis of gold nanoparticles. Different bioactive constituents such as triterpenoid, saponin, tannin, ellagic acid, gallic acid and proanthocyanidines are present in *Terminalia arjuna* bark extract had been reported [23]. In ayurveda *Terminalia arjuna* is considered as miracle herb used for the treatment of cardiovascular and

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oxidative stress mediated disorders [24]. It is one of the most versatile medicinal plants having a wide spectrum of biological activity such as anti-dysentric, anti-pyretic, astringent, cardiotonic, lithotriptic, anti-coagulant, hypolipidemic, anti-uremic [25] anti-oxidative, anti-microbial [26] and nephroprotective [27] properties.

Complete blood count is essential to determine the types and number of cells in the blood. Assessment of standard hematological parameters i.e. Red Blood Cells (RBCs), White Blood Cells (WBCs), Hematocrit (HCT)%, Hemoglobin (HB), Platelets (PLTs) counts, Lymphocyte percentage, Red blood cell Distribution Width (RDW) % and, Platelet Distribution Width (PDW)% are valuable indicators that help in diagnosing disease conditions such as anemia, infection and many other disorders.

Therefore, the present study has been focused on the characterization of green synthesized gold nanoparticles using aqueous bark extract of *Terminalia arjuna* and to evaluate whether this herbal nanoparticle can increase the efficiency of herb for alteration of hematological indices against acetaminophen induced toxicity in male Wistar rats.

Materials and Methods

Chemicals

Chloroauric acid ($HAuCL_4$) was purchased from sigma Aldrich (Lot#MKBZ67464, CAS: 16903-35-8$HAuCl_4$), Ethylene diamine tetra acetic acid (EDTA).

Green synthesis of AuNPs using bark extract of *Terminalia arjuna*

Terminalia arjuna was collected from Laterite region of Gope Palace (Raja N.L Khan Women's College) Medinipur, Paschim Midnapore district, West Bengal, India. Taxonomic identification was done by Department of Botany, Raja Narendra Lal Khan Women's College (Autonomous).

The bark samples was cut into small pieces and washed in running water to remove dirt and adhering debris. Then it was washed with ethanol to sterilize the samples. Then samples were dried under shade. Then the dried barks were ground into fine powder. 1 gm of powdered barksample was dissolved in 100 ml of distilled water by using magnetic stirrer and incubated at 50-60^{0}C for 15 min. The obtained crude extract was filtered by using Whatman No.1 filter paper and then filtrate was collected into 250 ml Erlenmeyer flask and stored at room temperature.

For green synthesis of AuNPs, 10 ml aliquot of bark extract was mixed with 100 ml of 1 mM $HAuCl_4$ solution and the mixture was stirred for 10 min at 60-70^{0}C for AuNPs synthesis. The change in color mixture was noted by visual examination. Then the green synthesized gold nanoparticles were lyophilized for freeze drying to get powder form. Then the green synthesized AuNPs powder was stored in air tight container.

Characterization of green synthesized AuNPs

UV- Visible Spectrophometer (UV-2450, Shimadzu, Japan) was utilized to measure Surface Plasmon Resonance (SPR) absorption band of green synthesized gold nanoparticles at wavelength of 400-800 nm. Field emission scanning electron microscope (FESEM) (Carl Zeiss, Germany) used to determine the shape and morphology [28] of the green synthesized AuNPs. High resolution transmission electron microscopy (HRTEM) (JEOL JEM 210) was operated for size and surface morphology analysis of the nanoparticles. HRTEM was equipped with energy dispersive X-ray spectroscopy (EDX) to study the elements composition and estimate their proportions. In addition, the selected area electron diffraction (SAED) pattern was also conducted. Fourier transform infrared spectra (FTIR) of *Terminalia arjuna* bark extract and green synthesized AuNPs was measured using (FTIR- NICOLET 6700) with resolution 4 cm^{-1} and scanning range between 4000 to 500 cm^{-1}. The crystalline nature of green synthesized AuNPs was determined by X-ray diffraction (XRD-Bruker, D2 PHASER) pattern operating at a voltage 30 kV and current 10 mA with Cu-Kα radiation. The size and dispersity of the nanoparticles in medium was using dynamic light scattering (DLS) (Zetasizer Ver. 6.20, Malvern Instruments, and UK).

Selection of animals

The experiment was conducted on 24 healthy male Wistar rats obtained from authorized Chakraborty Animal suppliers, Kolkata (M/S Chakraborty Enterprise Registration no.: 1443/PO/b/11/CPCSEA). The rats were nearly of same age, weighing 100-120 g. Animals were housed in appropriate cages three rats/cage at standard laboratory condition in a well-ventilated room with controlled temperature(25 ± 4^{0}C, humidity: 50 ± 10% and 12 h day/night cycle). They were provided with standard food and water ad libitum.

Treatment schedule

The rats were divided into four groups, each groups having six rats (n = 6): Group-I: Control; Group- II: received acetaminophen intraperitoneally at concentration 500 mg/kg of body weight for 14 days; Group-III: received intraperitoneal infusion of acetaminophen (500 mg/kg/day) along with co-administration of *Terminalia arjuna* bark extract (175 µg/kg/day) for 14 days; Group-IV: received intraperitoneal infusion of acetaminophen(500 mg/kg/day) along with co-administration of green synthesized AuNPs (175 µg/kg/day) for 14 days.

After the treatment the animals were anesthetized by inhalation of chloroform and then sacrificed for blood collection. The whole blood was collected in polypropylene tubes containing Ethylene diamine tetra acetic acid (EDTA) for haematological assessment. In this study all the experimental protocols were approved by the Animal Ethical Committee(Reference number: 02/IAEC(3)/S/RNLKWC/2017) and were maintained as per Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India (Registration no.: 1905/PO/Re/S/2016/CPCSEA).

Hematological analysis

Blood count was performed by Semi-Automatic Hematology Analyzer (ABACUS Vet 5, Diatron) to determine different haematological parameters such as Red Blood Cells (RBCs), White Blood Cells (WBCs), Hemoglobin (HB), Hematocrit (HCT)%, Lymphocyte percentage, Platelets (PLTs), Red blood cell Distribution Width(RDW) % and Platelet Distribution Width (PDW)%. Hematological analysis was performed by using 25 µl whole blood sample. Electrical Impedance method was used for this analysis.

Statistical analysis

The data were calculated and statistical analysis was done by using statistical package, Origin 6.1, Northampton, Mass, USA. The statistically calculated data were expressed as Mean ± SE, n=6. Comparison was done between the means of control and with all experimental groups, by ANOVA followed by multiple twotail t-test. Bars for a specific data differ from each other significantly (p<0.05).

Results and Discussion

Characterization of green synthesized AuNPs

Visual observations: This preliminary phytochemical analysis showed the formation of green synthesized gold nanoparticles. When aqueous bark extract of *Terminalia arjuna* was added to chloroauric acid solution the color changed from pale yellow to ruby red color within a minute due to the excitation of surface Plasmon oscillation induced by electromagnetic field and reduction of gold ions (Figure 1). This indicated *Terminalia arjuna* mediated transformation of cholroauric acid into green synthesized AuNPs.

UV- Visible spectral analysis: The UV- visible spectral analysis showed a broad absorbance peak centered at 524 nm (Figure 2) due to size dependent quantum mechanical phenomenon called surface Plasmon resonance (SPR) [29]. When the SPR bands centred between 500 to 600 nm it confirms the formation of green synthesized AuNPs in the solution.

FESEM analysis: The FESEM study of green synthesized AuNPs confirmed that particles were mostly spherical in nature and had sizes less than 40 nm (Figure 3). The size distribution study also revealed that nanoparticles were well dispersed.

HRTEM with EDX analysis: High resolution transmission electron microscopy (HRTEM) study is an important device to analyze the accurate size and morphology of green synthesized AuNPs in the dry state. In HRTEM analysis, actual diameter of the nanoparticles was determined having size between 7-20 nm (Figure 4a-4c). In addition analysis through EDX spectroscopy confirmed the presence of elemental gold (Au) signal and consistent distribution of the green synthesized AuNPs (Figure 4d). The elemental composition analysis of EDX indicated strong signal for gold and weak signals for oxygen and carbon, which might be due to the bioorganic molecules bound to the surface of AuNPs. The SAED pattern with bright circular rings explains that nanoparticles were highly crystalline in nature (Figure 4e) [30].

FTIR analysis: FTIR spectrum envisages the molecular arrangements of different functional groups of aqueous *Terminalia arjuna* bark extract and colloidal AuNPs (Figure 5). This study helps to predict the potential biomolecules responsible for the reduction of Au^{3+}

Figure 1: Colour Change during phytoreduction of chloroauric acid to ruby red colour.

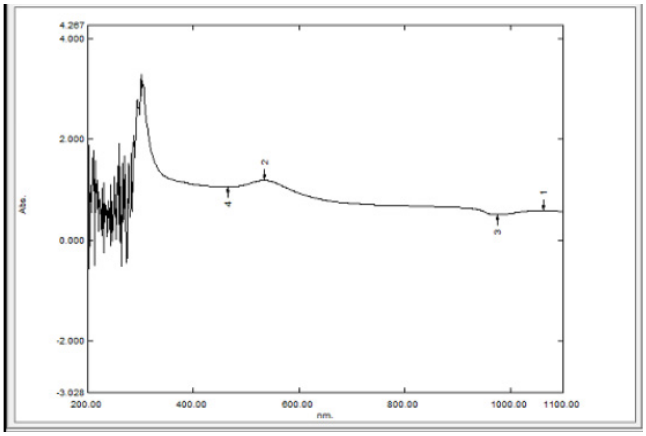

Figure 2: UV- spectra of *Terminalia arjuna* containing AuNPs.

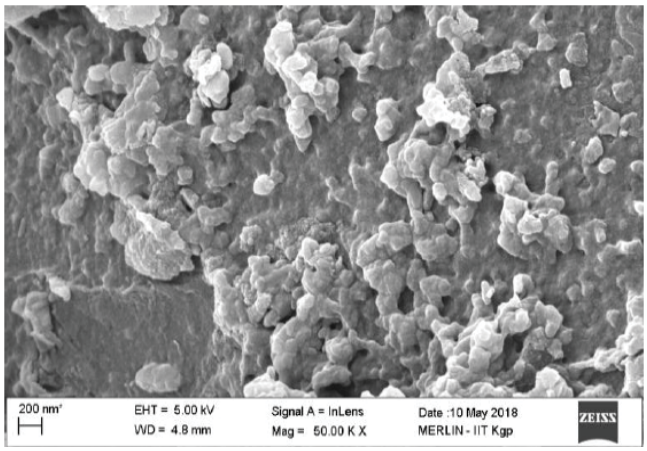

Figure 3: FESEM image of green synthesized AuNPs.

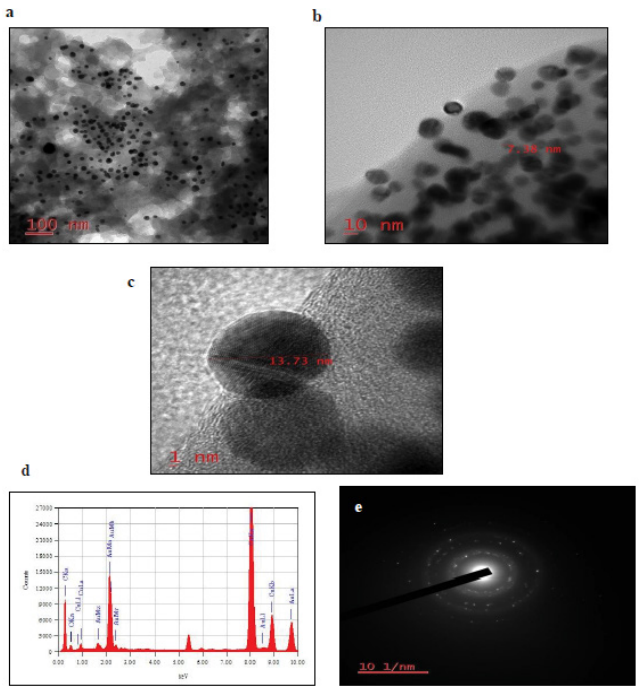

Figure 4: a-c- HRTEM images of green synthesized AuNPs with different magnification; d-EDX spectroscopy display chemical composition of green synthesized AuNPs. e-SAED pattern of green synthesized AuNPs.

ions and capping of reduced Au^0 NPs synthesized using *Terminalia arjuna* bark extract. The strong IR bands were observed at 3379.84, 2928.92, 1722.34, 1615.70, 1518.62, 1443.65, 1317.92, 1204.60, 1073.61, 816.67, 780.41, 627.88, 464.35 cm^{-1}. The strong peak at 3379.84 cm^{-1} corresponds to N-H stretching vibrations of primary amines. The band at 2928.92 cm^{-1} corresponds to C-H stretching of asymmetry and symmetry vibrations, respectively. Another peak at 1722.34 cm^{-1} belongs to C=O carbonyl stretching. The bands at 1615.70, 1518.62, 1443.65 cm^{-1} attributed to similar conjugation effects of N-H and C=c stretching vibrations. The IR bands observed at 1317.92, 1204.60, 1073.61 cm^{-1} may be ascribed to C-N and C-O-C stretching modes, respectively. The bands at 816.67, 780.41 cm^{-1} may be assigned to C-H bending. The band observed at 627.88 cm^{-1} may be due to the bending vibrations of N-H groups in protein. Hence, FTIR analysis indicates that terpenoid, saponins, tannins, flavonoids, glycosides and polyphenolic compounds are present in the bark extract of *Terminalia arjuna* [31]. The absorbance of IR band observed at 464.35 cm^{-1} is ascribed to the metal peak due to reduction of Au^{3+} ions to Au^0 of green synthesized AuNPs.

X ray diffraction pattern analysis: The XRD pattern showed four distinct diffraction peaks with 2θ values at 38.17^0, 44.370, 64.560, 77.540 which can be indexed to (111), (200), (220), (311) reflections of FCC (face centered cubic) structure of metallic gold respectively;(JCPDS No. 04-0784) revealing that the green synthesized AuNPs were composed of pure crystalline Au (Figure 6). In the literature similar results were reported for AuNPs synthesized using different biological entities [32].

Dynamic light scattering analysis

Particle size and dispersity of AuNPs generated and capped with aqueous bark extract of *Terminalia arjuna* were determined by DLS study [33]. In the present study, the size of the nanoparticles ranged from 7 to 90 nm with average range diameter of 20 nm (Figure 7). The measured size of the green synthesized AuNPs have larger hydrodynamic diameter due to the hydrated state of the sample in DLS method.

Hematological analysis

The present study was conducted to evaluate the protective effect of green synthesized AuNPs against acetaminophen induced hematological alterations in Wistar rats. Acetaminophen chemically named as N- acetyl p-aminophenol is widely used analgesic and anti-pyretic which at overdose can cause acute hepatic damage. The protective activity of the green synthesized AuNPs was obtained when co- administered once daily at a dose of 175 µg/kg body weight for 14 days with acetaminophen administration.

In this study, it has been observed that RBCs count significantly decreased after intraperitoneal administration of acetaminophen compared to control, indicating occurrence of anaemia and hypoxia (Figure 8). The decrease in RBCs count means destruction of Red blood cells and reduction in the rate of erythropoiesis. RBCs count conducted with co-administration of green synthesized AuNPs along with acetaminophen treatment showed significant increase compared to the acetaminophen treated rats but there was no effective alteration in RBCs count with co-administration of *Terminalia arjuna* extract compared to acetaminophen treated rats. Normally RBCs are produced from hemopoietic stem cells in bone marrow and undergoes maturation directed by erythropoietin. Thus, alteration in RBCs count might be due to hematopoietic system [34].

A decrease in the HB level and HCT% after acetaminophen

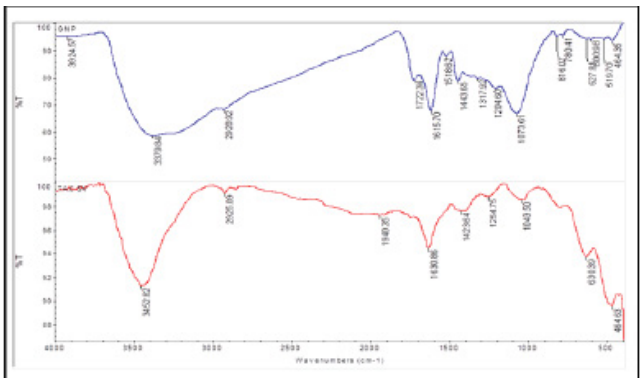

Figure 5: FTIR study of green synthesized AuNPs (blue) & *Terminalia arjuna* bark extract (red).

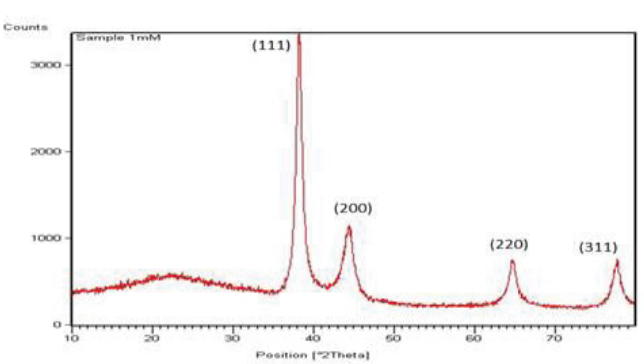

Figure 6: XRD study of green synthesized gold nanoparticles.

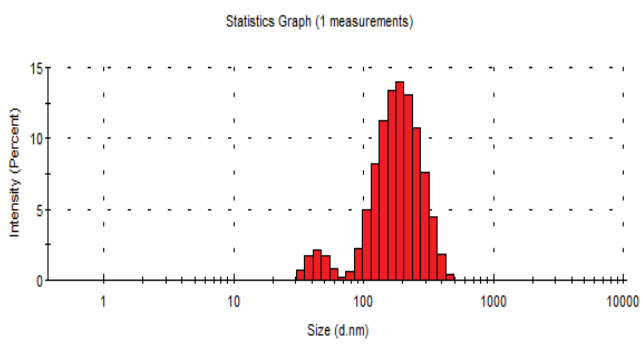

Figure 7: DLS study of green synthesized AuNPs.

treatment in contrast to the control rats was due to the destruction and decrease in production of RBCs. Haematocrit percentage or packed cell volume measures the volume percentage of red blood cells in blood. After co-administration of green synthesized AuNPs there was significant elevation in the HB level and HCT% compared to acetaminophen treated rats but with co- administration of *Terminalia arjuna* extract did not show effective alterations in HB level and HCT% contrast to acetaminophen treated rats (Figures 9 and 10).

Lymphocytes are the first line of immune defense against different infections. The lymphocyte percentage accompanied byacetaminophen administration significantly reduced compared to control, after co-administration of green synthesized AuNPs there was significantly increased level of lymphocyte percentage compared to acetaminophen treated rats but co-administration of *Terminalia arjuna* extract did not

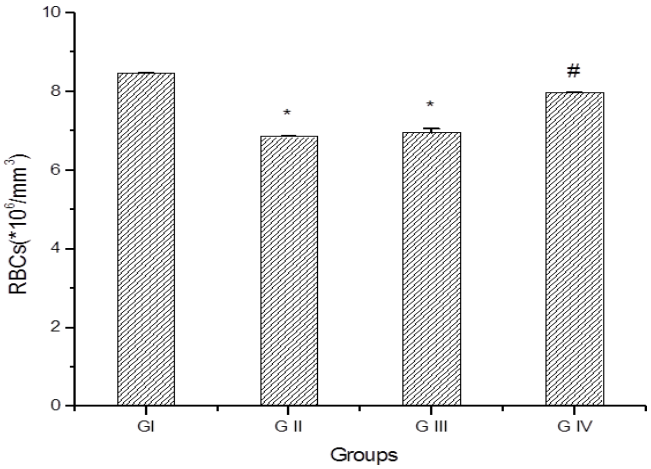

Figure 8: Effect of *Terminalia arjuna* bark extract and green synthesized AuNPs on RBCs count in rats after intraperitoneal administration of acetaminophen. Data are expressed as Mean ± SE (n=6).ANOVA followed by multiple two-tail t-test and data with different superscripts (*, #) in a specific vertical column differ from each other significantly (p<0.05). Groups, G I: control, GII: Acetaminophen (Uremic), G III: Acetaminophen + aqueous extract of TA, G IV: Acetaminophen+ green synthesized AuNPs.

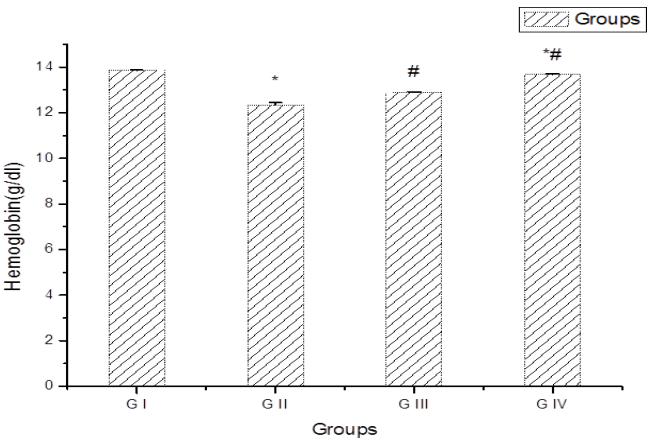

Figure 9: Effect of *Terminalia arjuna* bark extract and green synthesized AuNPs on Hemoglobin level in rats after intraperitoneal administration of acetaminophen. Data are expressed as Mean± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (*,#,*#) in a specific vertical column differ from each other significantly (p<0.05).Groups, G I: control, GII: Acetaminophen (Uremic), G III: Acetaminophen + aqueous extract of TA, G IV: Acetaminophen+ green synthesized AuNPs.

show effective change against acetaminophen treated rats (Figure 11). Hence, during toxicity or infections lymphocyte percentage declines. It has been reported that AuNPs directly interacts with lymphocyte [35]. This confirms the effectiveness of AuNPs as novel drug delivery system to the lymphocyte [36].

In, Figure 12 showed significant increase in the WBCs after acetaminophen administration compared to control, after co-administration of green synthesized AuNPs there was significantly decreased level of WBCs compared to acetaminophen and *Terminalia arjuna* extract treated rats. WBCs are the cells of immune system that are involved in protecting the body against infections. Hence, green synthesized AuNPs manifested effective protection of WBCs during infections.

There was significant increase in the RDW%, after acetaminophen

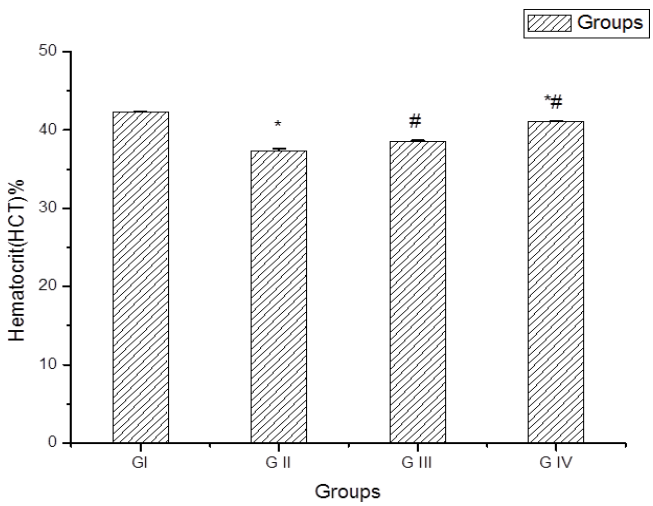

Figure 10: Effect of *Terminalia arjuna* bark extract and green synthesized AuNPs on Hematocrit % in rats after intraperitoneal administration of acetaminophen. Data are expressed as Mean± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (*,#,*#) in a specific vertical column differ from each other significantly (p<0.05).Groups, G I: control, GII: Acetaminophen (Uremic), G III: Acetaminophen + aqueous extract of TA, G IV: Acetaminophen+ green synthesized AuNPs.

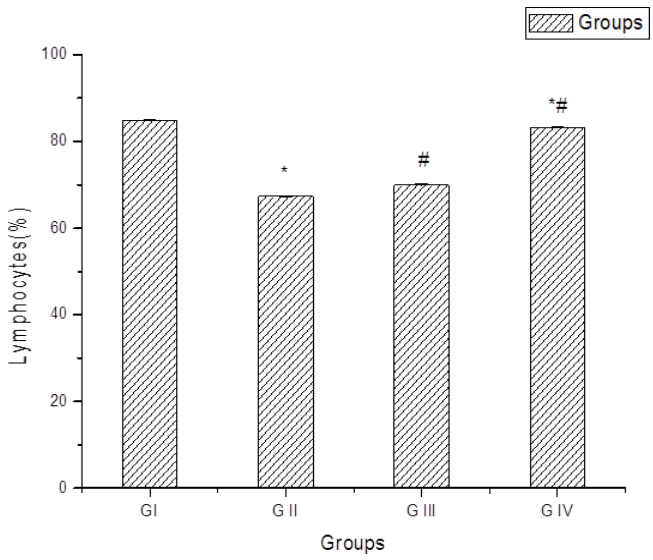

Figure 11: Effect of *Terminalia arjuna* bark extract and green synthesized AuNPs on Lymphocytes(%) in rats after intraperitoneal administration of acetaminophen. Data are expressed as Mean± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (*,#,*#) in a specific vertical column differ from each other significantly (p < 0.05).Groups, G I: control, GII: Acetaminophen (Uremic), G III: Acetaminophen + aqueous extract of TA, G IV: Acetaminophen+ green synthesized AuNPs.

treatment compared to control, respectively but co-administration of green synthesized AuNPs with acetaminophen showed significant decrease in red blood cell indices compared to acetaminophen and *Terminalia arjuna* extract treated rats (Figure 13). Red cell distribution width (RDW) test is the measurement of the range in the volume and size of erythrocytes. RBCs carry oxygen from lungs to every cell in the body. The increase in RDW than normal indicates anemia a possible problem with body function that in turn may affect oxygen getting to various parts of the body.

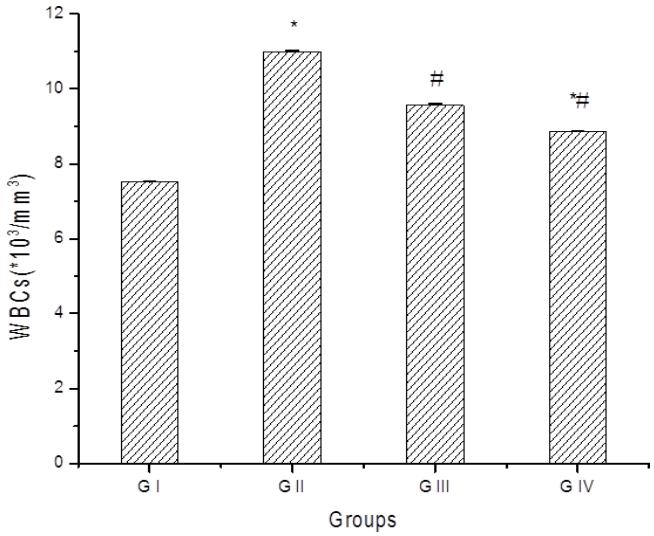

Figure 12: Effect of *Terminalia arjuna* bark extract and green synthesized AuNPs on WBCs count in rats after intraperitoneal administration of acetaminophen. Data are expressed as Mean± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (*,#,*#) in a specific vertical column differ from each other significantly (p<0.05).Groups, G I: control, GII: Acetaminophen (Uremic), G III: Acetaminophen + aqueous extract of TA, G IV: Acetaminophen+ green synthesized AuNPs.

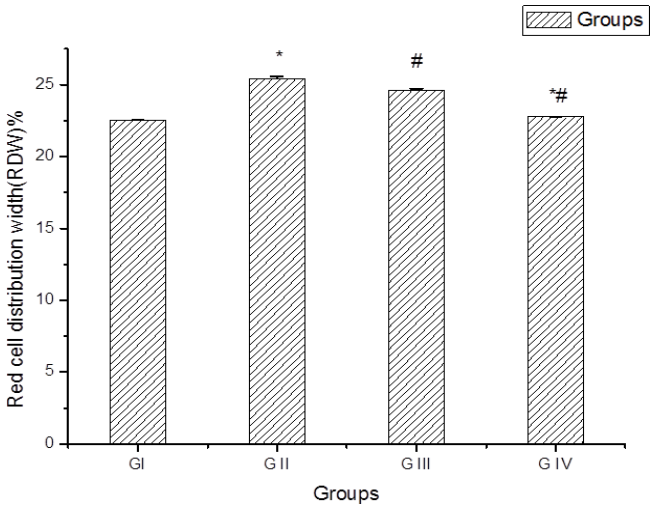

Figure 13: Effect of *Terminalia arjuna* bark extract and green synthesized AuNPs on RDW% in rats after intraperitoneal administration of acetaminophen. Data are expressed as Mean± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (*,#,*#) in a specific vertical column differ from each other significantly (p<0.05).Groups, G I: control, GII: Acetaminophen (Uremic), G III: Acetaminophen + aqueous extract of TA, G IV: Acetaminophen+ green synthesized AuNPs.

Figure 14, showed significant decrease in PDW% after intraperitoneal administration of acetaminophen compared to control, respectively but co-administration of green synthesized AuNPs with acetaminophen showed significant increase in PDW% compared to acetaminophen and *Terminalia arjuna* extract treated rats.

Platelets (PLTs) are small blood cells that help in clotting of blood. When bleeding occurs platelets rush to the site of damage and form sticky plug that repair the damage and stops the bleeding. Figure 15, showed PLTs count gets significantly elevated after intraperitoneal

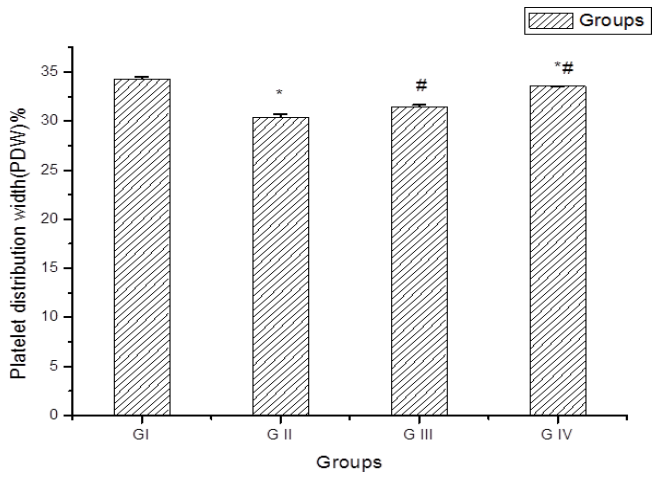

Figure 14: Effect of *Terminalia arjuna* bark extract and green synthesized AuNPs on PDW% in rats after intraperitoneal administration of acetaminophen. Data are expressed as Mean± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (*,#,*#) in a specific vertical column differ from each other significantly (p<0.05).Groups, G I: control, GII: Acetaminophen (Uremic), G III: Acetaminophen + aqueous extract of TA, G IV: Acetaminophen+ green synthesized AuNPs.

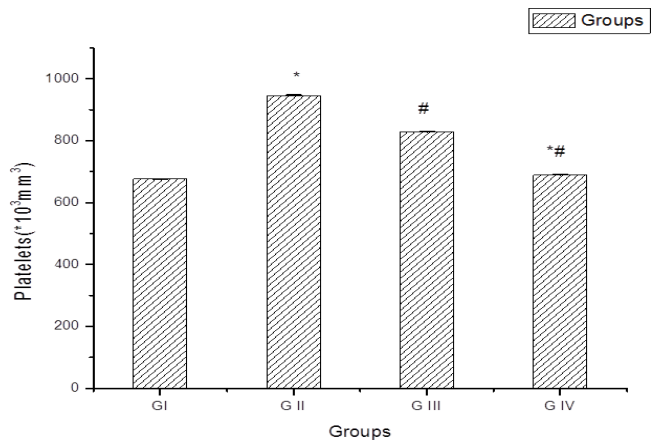

Figure 15: Effect of *Terminalia arjuna* bark extract and green synthesized AuNPs on Platelets count in rats after intraperitoneal administration of acetaminophen. Data are expressed as Mean± SE (n=6). ANOVA followed by multiple two-tail t-test and data with different superscripts (*,#,*#)in a specific vertical column differ from each other significantly (p<0.05).Groups, G I: control, GII: Acetaminophen (Uremic), G III: Acetaminophen + aqueous extract of TA, G IV: Acetaminophen+ green synthesized AuNPs.

administration of acetaminophen compared to control. The increase in PLTs might lead to thrombosis inside blood vessels and progression of atherosclerosis which is considered to be most toxic induced by acetaminophen. But after co-administration of green synthesized AuNPs with acetaminophen treatment showed effective significant decrease compared to acetaminophen and *Terminalia arjuna* extract treated rats.

Conclusions

The current investigation focuses on the effective role of green synthesized gold nanoparticles against acetaminophen induced hematological alterations in male Wistar rats. The AuNPs were synthesized using *Terminalia arjuna* bark extract, which acted as both reducing and capping agent. This greener herbal method

approach is eco-friendly, cost effective, rapid and also can be easily scaled up for large-scale synthesis. The finding results indicated that green synthesized gold nanoparticles might be a useful agent to restore hematological alterations. Hence, green synthesized AuNPs supplementation might serves as a harmless therapeutic approach for protection against infections.

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