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Diminazene aceturate modified nanocomposite for improved efficacy in acute trypanosome infection

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ABSTRACT

Objective: To investigate the improved antitrypanocidal activity and toxicity of diminazene aceturate modified Nano drug in experimental rats. **Methods:** Aqueous leaf extract of *Hyptis suaveolens* was used to reduce gold tetrachloride to its nanoparticle size and this was characterized and formulates with naturally synthesized polyhydroxybutyrateas a Nano carrier. A total of thirty (30) albino rats were group into 6 (A-F) of 5 rats each & infected intraperitoneally with 0.2 mL of the inoculum containing about 1×10^3 *Trypanosoma brucei brucei* parasites per 0.2 mL of blood. Groups A and B were treated with 3 and 6 minutes released orange PHB, Groups C and D were treated with 15 and 30 minutes released mango PHB formulated tablet while Groups E and F were negative (untreated) and standard drug (Dininazene aceturare) respectively. **Results:** The free drug and modified orange synthesized polyhydroxy butyrate shows antitrypanocidal activities by reducing the replicating rate of the parasite as compared to infect untreated. While the modified- mango synthesized shows increasing order of replication. There were significant increases in all the haematological parameter evaluated in the infected treated groups compared to infect untreated. But no significant difference ($P<0.05$) observed in the Catalase activity in the serum and liver of all the groups whereas, the modified orange synthesized shows significant decrease in other enzymes activities evaluated when compared with the free drug, mango synthesized and the infected untreated groups. **Conclusion:** Orange synthesized modified diminazene aceturate show efficacy as free drug with limited toxicity that can enhance the therapeutic.

1. Introduction

Drug deliveries have become important tools in the medical field due to the strong demand for the controlled delivery of pharmacologically active materials to cells, tissue, and organs. Many drug-delivery methods have been developed using polymers as drug carriers which can effectively deliver the drug to a target site and thus increase the therapeutic benefit, while minimizing side effects [1]. Because of the flexibility of polymers, it has become possible to engineer multiple

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functionalities required for efficient drug delivery, simultaneously maintaining biocompatibility, facile manufacturing, and stable formulation. Some of the traditionally used polymers like silicone have been suspected to cause cancer, therefore, there is a need for nontoxic, biodegradable, and biocompatible polymers.

However, one of the alternatives to conventional polymers is biodegradable biopolymer. Among the various biodegradable polymers, there is growing interest in the group of biopolymers known as polyhydroxyalkanoates (PHAs). Polyhydroxyalkanoate (PHA) is a polyesters class that is non-toxic, bio-derived and biodegradable. Polyhydroxybutyrateis one class of PHA that are insoluble in polar solvent, hence, relatively resistant to hydrolytic degradation. This differentiates PHB from most other biodegradable polymers, which are either water soluble or moisture sensitive. They have good oxygen permeability, ultra-violet resistance but poor resistance to acids and bases, biocompatible and hence are suitable for medical applications[2]. All these makes it a potentially good material for medical application (as bone plates, surgical sutures and blood vessel replacements), in the pharmaceutical and food industries, as biodegradable carriers for the long-term dosage of drugs, medicines, hormones, insecticides and herbicides [3].

African trypanosomiasis is one of the neglected diseases of sub-Sahara Africa that have impact of both human health and economic development of the affected areas [4]. The fight against the disease has relied mainly on vector control system and chemotherapies. However, the effectiveness and safety remains a source of concern due to the side effect associated with the drugs[5]. Diminazeneaceturate is a trypanocide used to treat animal trypanosomiasis, this drug has beenassociated with poor efficacy, limited solubility and major side effects[5] due to non-specificity of the drug on the target site.

The use ofemerging Nanotechnology such asgoldnanoparticles (AuNP) in area of medical applications especially as a drug carrier for targeted drug delivery has the advantage of bioavailability, in vivo stability, intestinal absorption, solubility, sustained and targeted delivery, and therapeutic effectiveness of several anticancer drugs(ref). Also, polymeric nanoparticles either synthetic or natural may act as efficient systems for drug release studies as these possess the property of biodegradability, biocompatibility andbiostable [6]. Due to these properties, they can overcome various barriers and target to the cancer cells without any harm to normal cells and tissues. Poly hydroxyl butyric acid (PHB) has also been used as a suitable nanoparticle for its biodegradability and biocompatibility in biomedical applications [7]. Therefore, this study is aim at synthesized gold nanoparticle functionalized with naturally synthesized polyhydroxybutyratefrom mango and orange seeds as a nanocarrier for diminazeneaceturate used for the treatment of animal trypanosome.

2. Materials and methods

2.1. Materials

2.1.1. Plant sample

Fresh leaves of hyptissuaveolens were obtained from Minna, Niger State Nigeria. Taxonomic authentications of the plants were carried out by Botanist at the Department of Biological science, Federal University of Technology, Minna. Nigeria. The leaves were destalked, washed with clean-water, dried at room temperature and finally grounded using a grinder mill.

2.1.2 . Experimental animals

Healthy albino rats of average weight 120-150 g were purchased from small animal holding unit, Federal University of Technology, Minna, Niger State Nigeria. The rats were maintained under standard laboratory conditions with unrestricted access to rat pellets and water ad-libitum. The study was carried out per the Guide for the Care and the Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, USA[8].

2.1.3. Reagent and assay kits

The gold chloride ($HAuCl_4$) was a product of Sigma Aldrich. The assay kits for AST, ALT, ALP and total protein were products of Randox Laboratories Ltd, United Kingdom. All other reagents used were of analytical grade and were prepared in distilled water.

2.2. Methods

2.2.1. Parasite strain

The parasite *Trypanosoma brucei brucei (T. brucei brucei)* was obtained from the Nigeria Institute for trypanosomiasis research (NITR) Vom Jos, Plateau State. The Parasite was maintained in the laboratory by serial blood passage into normal albino Rats until required.

2.2.2. Synthesis and characterization of gold nanoparticles

The synthesis and characterization of gold nanoparticles were as previously reported[9]. Briefly, one hundred milliliters of distilled water were added to 5 g of milled plant in an Erlenmeyer flask and then boiled for 5mins after which it was filtered, and then 0.5 mL of the plant extract was added to 9.5 mL of 1mM aqueous $HAuCl_4$ solution for the reduction of Au3+ ions. The synthesize gold nanoparticles were characterized with UV-VIS Spectrophotometer (UV-1800 Shimadzu).

2.2.3. Production and extraction of polyhydroxyalkanoate (PHA)

The production and extraction of PHA was as previously reported[10]. Briefly Production of PHA was carried out in a two-

stage fermentation process. In the first production stage, primary inoculum was prepared by culturing Bacillus megateriumin 100 ml of sterilized nutrient broth, at 37 ℃in an incubator shaker (New Brunswick Scientific, Innova 44) with a regulated speed of 120 revolutions per minute (rpm) for 24 h. In the second stage, 2 mL of the 24 h culture wasintroduced in to 100 ml of two sterilized nutrient deficient media (pH 7.0). The cultures were then incubated at 37 ℃ in a shaker (New Brunswick Scientific, Innova 44) at 120 rpm for 48 h. Extraction and quantification was done per the methods described in[11] with slight modifications.

2.2.4. Functionalization of gold nanoparticle

The functionalization of the gold nanoparticle was carried out using poly hydroxyl butyrate synthesized from orange and mango seed kernel as a nanocarrier.

2.2.5. Drug conjugation and release

Diminazeneaceturate was added to polyhydroxybutyrate (PHB) functionalized gold nanoparticle in aqueous phase and stirred for 30 minutes at room temperature [12]. All the formulations were made in to tablet form and left for 24 hours at room temperature to enable digestion of the mixture, and properly air dried. The tablets were assessed in deionized water based on in vitro dissolution method for 3 and 6 minutes (O-PHB), and 15 and 30 minutes of M-PHB). The absorbance of each solution was determined spectrophotometrically.

2.2.6. In vivo antitrypanosomal analysis

A total of thirty (30) albino rats were group into 6 (A-F) of 5 rats each & infected intraperitoneally with 0.2ml of the inoculum containing about 1×10^3 *T. brucei brucei* parasites per 0.2 mL of blood. Infection was monitored after 72 hours for the appearance of parasites in the newly infected animals [13]. Groups A and B were treated with 3 and 6 minutes released orange PHB, Groups C and D were treated with 15 and 30 minutes released mango PHB formulated tablet while Groups E and F were negative (untreated) and standard drug (DininazeneAceturare) respectively. Parasite count was done on daily basis per the method described by Ekanem & Yusuf[14]. The procedures described by Akanji *et al.*[15] were adopted for collection & preparation of serum and tissue homogenates.

2.2.7. Determination of biochemical & hematological parameters

The biochemical analyses were determined for alkaline phosphatase (ALP) based on methods of Tietz [16], Aspartate transaminase (AST) and alanine transaminase (ALT) as described previously [17]. The total protein concentration was estimated by biuret method as described by Sulaiman and Adeyemi [18], while catalase was estimated as described[19]. The Sysmex Haematology Systems (SysmexAmerica Inc., model no. KX-21N, Kobe, Japan) was used to determine the levels of haemoglobin (Hb), packed cell volume (PCV), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), neutrophils, lymphocytes and platelets [20].

2.2.8. Statistical analysis

Data analyses were performed using SPSS software (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL). All data are expressed as mean ± SEM. Analysis of variance (ANOVA) was used to test for differences between the groups. Duncan's multiple range tests was used to determine the significance of differences among the mean values at the level of $P<0.05$ [21].

2.3. Ethical approval

The principles governing the use of laboratory animals as laid out by the Federal University of Technology, Minna Committee on Ethics for Medical and Scientific Research and alsoexisting internationally accepted principles for laboratory animal use and care as contained in the Canadian Council on Animal Care Guidelines and Protocol Review were duly observed.

3. Results

3.1. Characteristic of Biosynthesis of gold nanoparticles

The biosynthesized gold nanoparticle of aqueous leaf extract of Hyptissuaveolens had a characteristic brownish colour. UV-Visible spectrophotometric scanning showed that the biosynthesized gold nanoparticle had absorption peak of 535 nm (Figure 1). The Zetasizer measurement showed the average particle size of 28.5 nm with the intensity of 17.5%. TEM images showed shape to be spherical hexagonal and triangular at 50 nm. Energy-Dispersive Spectroscopy Analysis confirmed the presence of gold and other elements (carbon, copper and oxygen). The maximum optical adsorption peak was observed at approximately 2.30 keV and addition signals for carbon at 0.20, oxygen 0.50 and copper 8.30. The FTIR spectra reveals a strong band in functional group region at 3448.84 cm-1 and this strong band region corresponds to the hydroxyl group and the finger print region at 1643.41 cm^{-1} corresponds to the alkene (C=C) and carbonyl (C=O) group.

3.2. Antitrypanosomal

The parasitaemia of infected untreated group increased infinitely while infected rat treated with orange based polyhydroxy butyrate nanodrug at 3 and 6 minute –treated group shows a decrease in the proliferation and complete parasite clearance on day 7 (Figure

2). Although rat treated with mango based polyhydroxy butyrate nanodrug at 15 and 30 minutes shows low replication of parasite compared with the untreated rats there was also a progressive increase in parasite count.

3.3. Biochemical parameters

The ALP and ALT activities were significantly lowered (P< 0.005) in serum and liver of rat treated with 3 and 6M O-PHB compared with the rats treated with free drugs and the untreated control. The serum AST in infected un-treated rats were significantly lowered (P<0.05) than all treated rats. However, serum AST activities in rat treated with O-PHB compared well with that of the rats treated with free drug. However, no significant (P<0.05) difference were observed in serum total proteins among the experimental groups (Figure 3).

The liver AST activities were significantly (P<0.05) higher in rats treated with 15 and 30-minute M-PHB than those treated with the free-drug and the untreated control. However, serum AST activities in infected-untreated rats were significantly (P<0.05) higher than all treated groups. The liver ALT activities in infected-untreated rats were significantly (P<0.05) higher than all treated groups. ALP activities were significantly (P<0.05) raised in liver and serum of rat treated with 15 and 30 minutes released of functionalized mango PHB when compared with the infected untreated controls. The liver total proteins in rats treated with 15 minutes released of functionalized M-PHB was significantly (P<0.05) higher than the controls (untreated and free-drug). However, no significant (P>0.05) differences were observed in serum total proteins and serum ALT activities in rats treated with 15 and 30 minutes released of functionalized M-PHB compared with the controls (untreated and free-drug) (Figure 4).

The serum and liver catalase activities in infected untreated rats were significantly (P< 0.05) higher than all treated groups. However, there were significant decrease (P< 0.05) in serum and liver catalase activities in rats treated with 3 and 6 minutes O-PHB compared with those treated with free-drug (Figure 5).

3.4. Hematological Parameters

Table 1 shows the level of haematological components in *T. brucei brucei* infected rats treated with diminazeneaceturate (freed rug) andorange - polyhydydroxy butyrate functionalize drug (M-PHB). There were significant increases (P<0.05) in the values of Hb, MCHC, PCV, RBC, and WBC in *T. brucei* infected rats treated with free drug andO-PHB when compared with untreated rats. However, except for the increase in WBC count in rats treated with 6 minutes O-PHB, the haematoalogical parameters compared well (P>0.05) between rats treated with free drug andO-PHB functionalize drug. Similarly, there were significant increases (P<0.05) in the values of Hb, MCHC, MCV, MCH, PCV, RBC, and WBC in *T. brucei brucei* infected rat treated with free drug andM-PHB when compared with untreated rats. However, RBC counts in rats treated with M-PHB (15 and 30-minute release) were significantly lowered while WBC count was significantly raised when compared with rats treated with free drug.

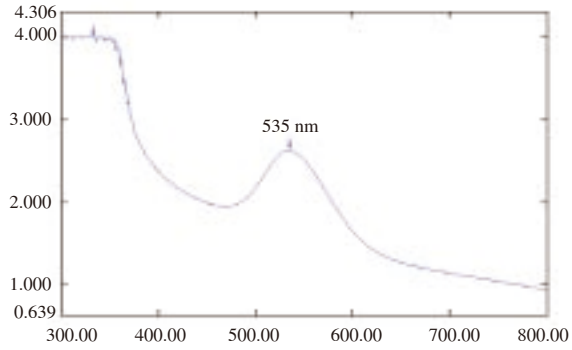


Figure 1. Absorption peak of biosynthesized gold nanoparticle, showing absorption peak at 535 nm.

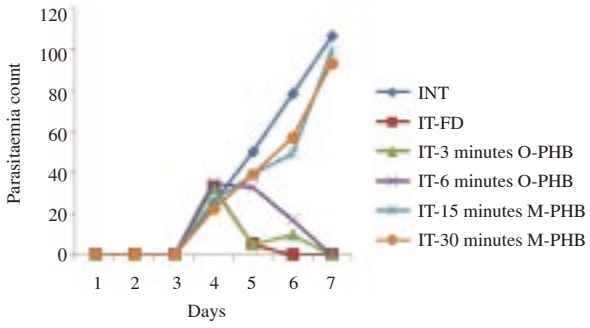


Figure 2. Parasite count of *T. brucei brucei* infected rat treated with orange based polyhydydroxy butyrate nanodrug.
Each point is an average count from five infected rats.

Table 1

Haematological components in *T. brucei* infected rats treated with orange- and mango- polyhydydroxy butyrate functionalize diminazeneaceturate.

GROUPS	Hb(g/dL)	PCV(%)	MCV(fL)	MCH(pg)	MCHC(g/dL)	RBC(10^9/L)	WBC(10^9/L)
IUT	6.33±1.52^a	19.00±4.58^a	8.37±2.40^a	2.78±0.80^a	33.31±0.00^a	3.33±1.76^a	12.00±2.51^a
IT-FD	14.46±0.35^b	44.66±0.88^b	52.33±0.88^b	16.00±0.57^b	32.00±0.00^a	8.10±0.25^b	11.33±1.85^a
IT-3 minutes O-PHB	11.23±0.12 ^{b}	35.33±0.88^b	55.33±0.88^b	17.33±0.33^b	31.33±0.33^a	7.50±0.74^b	10.46±0.74^a
IT-6 minutes O-PHB	12.83±1.11 ^{b}	40.33±4.17^b	55.66±2.33^b	18.00±1.15^b	32.00±0.57^a	7.33±1.09^b	15.43±4.09^b
IT-15 minutes M-PHB	11.50±0.10 ^{b}	34.66±0.33 ^{b}	53.33±0.33^b	17.66±0.33^b	34.33±0.33^a	5.66±0.08 ^{ab}	15.10±0.66^b
IT-30 minutes M-PHB	11.30±0.17 ^{b}	34.00±0.57 ^{b}	53.66±0.33^b	17.33±0.33^b	33.00±0.57^a	5.60±0.05^{ab}	15.96±0.20b

Mean ± SEM (n=5) followed by different superscript letters in a column are significantly different at P<0.05. IUT: Infected untreated, IT-FD: Infected treated with standard drug, IT-3 and 6 minutes O-PHB: Infected treated with 3 and 6 minutes released of functionalized orange PHB, IT-15 and 30 minutes M-PHB: Infected treated with 15 and 30 minutes released of functionalized orange PHB.

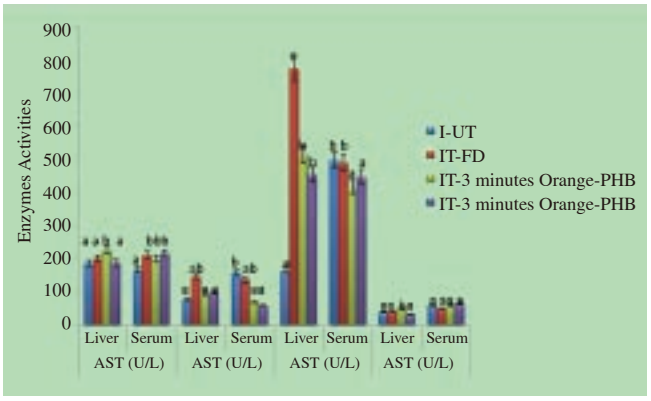


Figure 3. Serum and liver biochemical parameters in rats treated with O-PHB Nano-formulated.

Each bars represents mean ± SEM of five determinations. Bars with different superscript alphabet are significantly different (P<0.05).

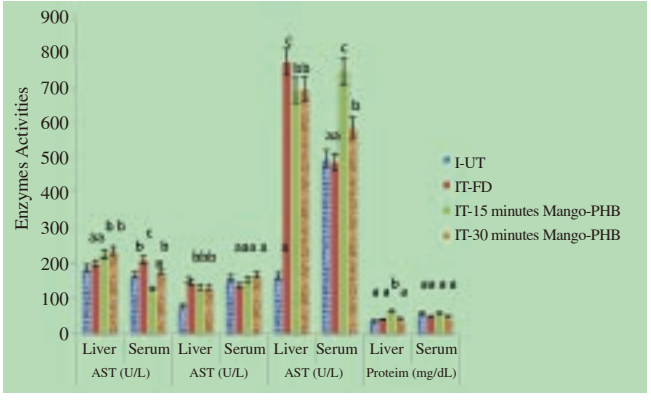


Figure 4. Serum and liver biochemical parameters in rats treated with M-PHB Nano-formulated.

Each bars represents mean ± SEM of five determinations. Bars with different superscript alphabet are significantly different (P<0.05).

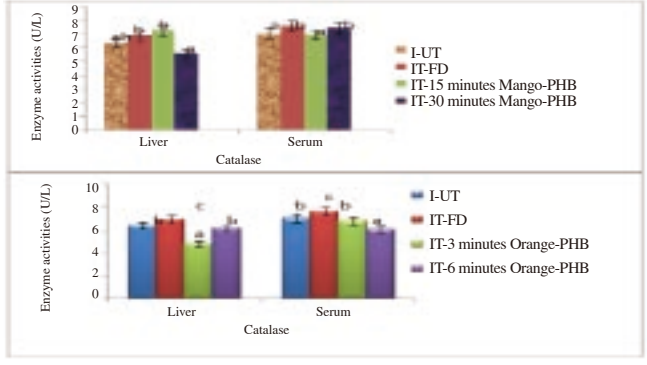


Figure 5. serum and liver catalase activities in rats treated with Nano-formulated.

Each bars represents mean ± SEM of five determinations. Bars with different superscript alphabet are significantly different (P<0.05).

4. Discussion

The development of new therapeutic or improving existing ones against trypanosomes is an important subject and it has been a subject of some recent investigations, mainly due to the shortcoming of the available therapy [22]. Nanotechnology is a powerful tool that can be used to reduce the toxic side effect of drug therapy.

In this study, free diminazeneaceturate and the PHB-Nano formulateddiminazeneaceturate were evaluated for comparative antitrypanosomal activities in order to determine whether conjugating drugs to surface PHB- nanoparticles could optimize drug delivery to target site while minimizing collateral damage. The parasitaemia count obtained from treatment with diminazeneaceturate showed the drug produce satisfactory efficiency in clearing the parasite from circulation, however, the unwanted deleterious effect of this drug is our major concern. The delayed in parasite clearance to day 7 post infection following treatment with O- PHB Nano formulated drug does not translate to reduced efficacy of the drug by Nano encapsulation but, rather, indicate gradual release of the drug from the formulation into the target site. This is an essential mechanism used by nanoparticles to improving efficacy and reduced toxicity of the encapsulated drug.

The liver is known to play vital role in intermediary metabolism of biomolecules and its cellular integrity can be altered by toxic side effect of drugs, chemicalsand related disease [23]. Evaluation of biochemical indices in the liver and serum are useful ‘markers’ for assessing clinical symptoms and tissue damages produce by drug or toxicant [24]. Alkaline phosphatases are often used to assess the integrity of plasma membrane and endoplasmic reticulum AST and ALT are liver based enzymes that catalyze transamination reaction[25]. However, high activities of these enzyme in serum occur during liver damage when there are leakages from their based into blood circulation. These enzymes therefore provide significant information about the integrity of liver.In the present study, the treatment of rats with diminazeneaceturate significantly raised the levels of ALP and ALT enzyme in rats. Such elevation in the enzymesactivities willadversely affect the metabolism ofamino acid and carbohydrate with consequent effect on ATP generation[26]. Interesting, the treatment of rats with 3 and 6 minutes O-PHB Nano formulated drug decreased the elevated levels of ALP and ALT enzyme significantly. In contrast, AST andALP levels significantly increased in treated with 15 and 30 minutes M-PHB when compared with those treated with free drug. This finding shows that PHB synthesize from orange seed improved efficacy and reduced toxicity of diminazeneaceturate, than thePHB synthesize from mango seed. According to Yang *et al.*, [27], liver is one of the frequent target organs and a central site of accumulation of nanoparticles, thus the alterations to the levels of enzymes recorded in this may represent adaptive mechanisms by the animals to offset stress induced by exposure to the M-PHB Nano formulated drug. Total protein is composed of albumin and globulin and play important roles in determining the synthetic and excretory roles of the liver and kidney [13]. The significant decrease in total protein in rats treated with M-PHB Nano formulated drug could be as a results of increased anabolism, decreased catabolism or malabsorption by the liver as an

adaptive mechanism to overcome the stress imposed by the M-PHB Nano formulated drug. Previous study [28] has demonstrated that administration of drugs or chemical agents could enhance protein synthesis de novo as part of adaptive mechanisms.

High level of catalase activities has been reported to correlate with severity of oxidative stress during trypanosome infection[29]. The results of the effect of the Nano formulation on catalase activities showed that the rat treated with O-PHB Nano formulated drug had lower catalase activities than those treated with the free drug and the untreated rats. This is an indication that O-PHB Nano formulated drug exert more therapeutic efficiency in alleviating the trypanosomes-induced free radical's generation. However, this efficiency was not demonstrated by mango based-PHB Nano formulated drug.

Hematological indices generally provide information on the deleterious effects of trypanosome infection and productive performance of the infected animals [18]. The significant decreases in the values of Hb, MCHC, PCV, RBC, and WBC count in *T. brucei brucei* infected untreated rat is an indication of anemic condition [30]. This finding agrees with study of Ekanem *et al.*[31], who reported that trypanosome infection cause anemia as a result of massive erythrophagocytosis by an expanded and active mononuclear phagocytic system (MPS) of the host. It has also been established that trypanosomes infection result in depletion of reduced glutathione on the surface of the red blood cell they're by increasing the susceptibility of red blood cell membrane to oxidative damage [29]. These hematological parameters compared well between rats treated with free drug and O-PHB functionalize drug. However, contrary to the report of [18], the present study showed that administration of free diminazeneaceturate does not modulate the state of anemia and immunosuppressive conditions in trypanosome infected rats

In contrast, RBC counts in rats treated with M-PHB were significantly lowered while WBC counts were significantly raised when compared with rats treated with free drug (Table 1).WBCs are known for their defensive role against foreign substances and infectious agentsthrough the production, transportation and distribution of antibodiesin immune response [32]. The significant increased WBC counts following treatments with M-PHB reflect leucopoetin-release and possible immunomodulatory effects of the nanoparticle which augmented the production of WBC to overcome the stress induced by the constituent of the Nano formulation. This will enhance the capabilityof generating phagocytes and antibodies against the recognizable antigens from subsequent challenge [33,34].

Conclusion

Hyptis suaveolens plant shows the ability to reduce gold chloride to gold nanoparticle which confirms the potential value of the plant in nanoparticle synthesis. Noteworthy, this study has demonstrated the fact that orange based polyhydroxy butyrate modified gold nanoparticles could be exploited for drug delivery and/or used tomodulate toxic virtues of diminazeneaceturate. However, the increase toxicity demonstrated by orange based polyhydroxy butyrate Nano formulation called for thorough screening of natural products for use as a carbon source in the synthesis of polyhydroxy butyrate for effective drug delivery

Acknowledgments

Conflict of interest statement

The authors declared no conflict of interest exist

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