

TOXICOLOGICAL AND ANTI-TRYPANOSOMAL EVALUATION OF POLYMER CONJUGATES OF EFAVIRENZ, TENOFOVIR, AND DOLUTEGRAVIR

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ABSTRACT

Background and purpose: Trypanosomes are parasitic protozoa that cause Chagas disease in central and South America and sleeping sickness in sub-Saharan Africa leading to morbidity and mortality of millions of people. *T. brucei gambiense* accounts for more than 95% of reported cases and endemic in 36 sub-Saharan African countries. The of the study is to determine the toxicological and anti-trypanosomal evaluation of polymer conjugates of efavirenz, tenofovir, and dolutegravir. **Method:** Toxicity testing and LD50 determination of the Conjugates were carried out using lorke's method and after which anti-Trypanosoma evaluation was carried out by using a seven-day Trypanosoma brucei suppressive test in albino rat. Statistical analysis using one way ANOVA was carried out where differences with values of $p < 0.05$ were considered statistically significant. **Result:** From the lorke's method carried out, Doses of the drug conjugates of 10 -5000mg/kg witnesses no mortality within 24 hours indicating the safety of the drug conjugates (Tenofovir-Chitosan, Efavirenz-Chitosan and Dolutegravir-Alginate). Histological investigation of the Wister rat kidney and liver used for the toxicological study, revealed healthy kidney glomeruli and capillaries and healthy liver hepatocytes at doses between 10-4000mg/kg but at 5000mg/kg, the architecture of the liver revealed mild infiltrating leukocytes and piecemeal necrosis with sinusoid hepatocytes. The drug conjugates (Efavirenz-chitosan, Tenofovir-chitosan and Dolutegravir-alginate) screened against the Trypanosoma parasites had a significant increased ant-Trypanosoma activity compared to the drug alone (Tenofovir, Efavirenz and Dolutegravir), this might be probably due to the synergistic effect of the polymer used in the conjugation. **Conclusion:** the present study showed that the conjugates of dolutegravir, efavirenz and tenofovir may be considered as comparatively safe of toxicity as none of the conjugates produced any haematological challenge and no distortion of the architecture of the tissue. The study corroborated the application of the drug conjugates as better antitrypanosomal agent with lowest toxicity effect when compared with the melarsoprol, eflornithine, pentamidine and suramin.

KEYWORDS: Efavirenz, Tenofovir, Dolutegravir, Chitosan, Alginate, trypanosomes and Lorkes.

BACKGROUND

Trypanosomes are parasitic protozoa that cause Chagas disease in central and South America and sleeping sickness in sub-Saharan Africa leading to morbidity and mortality of millions of people.^[1] *T. brucei gambiense* accounts for more than 95% of reported cases and endemic in 36 sub-Saharan African countries.^[2] *Trypanosoma brucei*

gambiense and *T. b. rhodesiense* are the etiological agents of sleeping sickness in humans in sub-Saharan Africa. The parasites live and multiply extracellularly in blood and tissue fluids of their human host and are transmitted by the bite of infected tsetse flies (*Glossina* spp.). Human African Trypanosomiasis (HAT) currently affects 3,500 people/year; most patients live in the Democratic Republic of the Congo and an estimated 70

million people remain at risk of infection in sub-Saharan Africa.^[3] It is a neglected disease and drugs in use have serious toxic effects.

The cause of sleeping sickness differs depending on the subspecies. Infections with *T. b. rhodesiense* lead to an acute form of the disease while infections with *T. b. gambiense* give rise to a chronic infection. The symptoms of the first stage of the disease, defined by the restriction of trypanosomes to the blood and lymph system, include fever, headache, joint pain and itching.^[2,3] The clinical signs of the second stage of the disease, characterised by the invasion of trypanosomes into the central nervous system, are neurological and endocrinal disorders.^[4]

AIM OF THE STUDY: To determine the toxicological and anti-trypanosomal evaluation of polymer conjugates of efavirenz, tenofovir, and dolutegravir.

METHOD

2.1 Toxicity Testing and LD₅₀ determination of Conjugates

Lorke's method

Two phases were employed which are phase 1 and phase 2 respectively,

Phase 1: Nine Albino rats were utilised they were divided into three groups of three rats each. Each group of rats were administered different doses (10, 100 and 1000 mg/kg) of test substance. The rats were placed under observation for 24 hours to monitor their behaviours as well as mortality.

Phase 2: This phase involves the use of three rats, which were distributed into three groups of one rat each. The rats were administered higher doses (1600, 2900 and 5000 mg/kg) of test substance and then observed for 24 hours for behavior as well as mortality.

Then the LD₅₀ was calculated by the formula:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

Do – Highest dose that gave no mortality

D100 – Lowest dose that produced mortality (Joseph et al., 2019).

Haematology and Histology studies

After necropsy, samples from liver and kidney were obtained and fixed in buffered formalin (10 %) for at least 24 hours. The samples were dehydrated in graded series of ethanol, cleared in xylene, embedded in paraffin wax and sectioned at 4-5 μm. The slides were stained with the Hematoxylin and Eosin (H&E Stain). Changes were graded as follows: (0) showing no changes,^[1] for mild changes (less than 30%),^[2] moderate changes (more than 30% and less than 50%) and (3) for severe changes (more than 50%), following guidelines from the INHAND Project International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice.^[4]

Microscopy

To analyze the slides, photonic microscope from Leica (Leica, microsystems, Wetzlar, Germany), DM 750 and Lasez software was used to capture and store the images.

Biochemical and Haematological Study

On day 28, after anesthesia, blood samples were taken from the heart of the animal. Serum was obtained for biochemical analysis after centrifugation at 2500 rpm by min for 15 min. The biochemical indices such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) transaminases and haematological parameters like red blood cell (RBC), platelet crit (PCT), white blood cell (WBC) etc., were analysed. They were assessed in the laboratory of Biochemistry in Federal Medical Centre Keffi using diagnostic kits purchased from Roche Diagnostics Ltd (Germany).

Statistical Analysis

Results of biochemical analysis (transaminases) were expressed as mean ± standard deviation (SD). The difference between the different groups were compared using test of variance (one way ANOVA). Differences with values of p<0.05 were considered statistically significant. Statistics were performed using GraphPad software.

2.2 Anti-Trypanosoma evaluation: 7-day *T. brucei* suppressive test in Rat

Test organisms: *Trypanosoma brucei brucei*

Cryopreserved stabilate of *Trypanosoma brucei brucei* was obtained from the cryobank maintained at the Vector and Parasitology Study Department of the Nigerian Institute for Trypanosomiasis Research, Kaduna, Nigeria. After thawing the stabilate at 37°C, trypanosomes were screened for viability by examining wet smears prepared from the stabilate in the light microscope at x 400 magnification. The presence of motile trypanosomes was taken as indication of trypanosome viability.^[5]

Donor animals

Two albino Wistar rats were utilised as donor animals. Each rat was intraperitoneally inoculated with blood suspension (inoculum) prepared from the thawed stabilate. The inoculum was prepared by the addition of normal saline to a small amount of the blood from the stabilate until the trypanosome count was 2 per microscopic field. The rats were inoculated with 0.2 ml of the prepared blood suspension. Parasitemia in the inoculated rats was monitored three days after inoculation to determine the establishment of active infection. Subsequently, the level of parasitemia was determined daily until parasite count was about was 10⁹ per milliliter of blood. Following infection with the trypanosomes, at which point the donor rats were sacrificed.^[5]

Determination of parasitemia

The trypanosome count in the infected rats was estimated by the rapid matching method of Herbert and Lumsden (1976). Briefly, a drop of whole blood collected by tail snip or cardiac puncture was placed on a clean grease free glass slide and a cover glass placed over it; the blood spread into a thin circular film. The slide was then placed under the light microscope and examined at x400 magnification. The distribution of trypanosomes among the red blood cells (RBCs) was matched against the Lumsden's chart and the approximate number of trypanosomes per milliliter of blood estimated.^[5,6]

Reconstitution of the Drug conjugates and reference drug

Solutions of the drug conjugates and drug (Diminazene aceturate) was reconstituted in the supplemented medium. Initially, 100 mg/mL stock solutions of the respective conjugates and standard drug were prepared; subsequently, the respective stock solutions were serially diluted to yield conjugates with concentrations ranging from 10 mg/mL to 0.3125 mg/mL.^[5,6,7]

In vitro assay of the drug conjugates (Drug incubation survival assay)

The Drug Incubation Survival Test (DIST) was used for this assay. One hundred microlitres (100 µL) of the reconstituted solutions of each of the conjugates, as well as the reference drug, were separately dispensed in duplicates into wells of a 96-well microtitre plate. Twenty microlitres (20 µL) of the blood suspension containing *T. b. brucei* was added to each of these wells, and gently mixed together. Control wells containing only 100 µL supplemented medium and 20 µL blood suspension were also included. The micro plate was place in a desiccator containing about 5% carbon dioxide and maintained at 37°C in an incubator. Wet smears were prepared from each of these wells, twenty (20) hours post-incubation. Each smear was examined in the light microscope (X 400 magnification) and the counts of motile trypanosomes were taken over three fields of

view, given a total of nine observations per concentration of drug/conjugates. Similarly, trypanosome counts were also taken for smears prepared from the control wells. Reduction in the number of motile trypanosomes compared to the control wells was taken as index for in vitro activity.^[6,7]

RESULT

3.1 Lorkes Method

Doses of the drug conjugates of 10 -5000mg/kg witnesses no mortality within 24 hours indicating the safety of the drug conjugates (Tenofovir-Chitosan, Efavirenz-Chitosan and Dolutegravir-Alginate, (Table 1).

3.2 Histological Investigation of the Wister Rat Kidney used for the Toxicological Study

A-F: Kidney glomeruli and capillaries revealing healthy architecture (black arrow). A-F: Kidney revealing mild infiltration of renal corpuscles with glomeruli tufts of capillaries (blue and green arrows) all at 10, 100, 1000, 1600, 2900 and 5000mg/kg Efavirenz-chitosan, Tenofovir-chitosan and Dolutegravir-alginate.

3.3 Histological Investigation of the Wister Rat Liver used for the Toxicological Study

A-F: liver revealing healthy hepatocytes (black arrow). A-F: Liver revealing mild infiltrating leukocytes and piecemeal necrosis with radiating cords of hepatocytes (blue arrow). A-F: Liver revealing mild infiltrating leukocytes and piecemeal necrosis with sinusoid hepatocytes (green arrow) all at 10, 100, 1000, 1600, 2900 and 5000mg/kg Efavirenz-chitosan, Tenofovir-chitosan and Dolutegravir-alginate.

3.4 Antitrypanosoma Screening of Drugs alone and its Conjugates

Drug conjugates were screened against the parasites with significant increased effect compared to the drug alone, this might be probably due to the synergistic effect of the polymer used in the conjugation (Tables 8 and 9).

Table 1: Lork's Method of Estimating LD₅₀.

DOSE Mortality within 24hours				
S/N	(Mg/Kg)	Efavirenz-Chitosan Conjugate	Tenofovir-Chitosan Conjugate	Dolutegravir-Alginate Conjugate
1	10	0/3	0/3	0/3
2	100	0/3	0/3	0/3
3	1000	0/3	0/3	0/3
4	1600	0/3	0/3	0/3
5	2900	0/3	0/3	0/3
6	5000	0/3	0/3	0/3

Table 2: Effect of Efavirenz-Chitosan Conjugate on Serum Renal Function Parameters.

Treatment	Dose (mg/kg)	Na (mmol/L)	K (mmol/L)	Cl (Mmol/L)	HCO ₃ (Mmol/L)	Urea (Mmol/L)	Creatinine(mg/dL)
Distilled water	5 ml/kg	139.25±1.38	4.15±0.26	100.2±2.14	26.50±1.71	4.30±0.35	0.70±0.09
Efavirenz-chitosan conjugate	10	135.50±2.60	3.50±0.25	93.7±2.78	27.50±1.32	4.55±0.55	0.63±0.09
	100	139.75±3.33	3.43±0.25	92.0±2.35	25.25±2.87	4.30±0.38	0.60±0.13
	1000	133.75±4.71	2.75±0.12*	89.2±0.63*	25.75±2.95	5.28±0.33	0.70±0.15

	1600	139.75±3.33	3.43±0.25	92.0±2.35	25.25±2.87	4.30±0.38	0.60±0.13
	2900	137.75±3.23	3.53±0.25	93.0±2.35	24.25±2.77	4.20±0.28	0.61±0.12
	5000	129.75±3.22	3.43±0.25	92.0±2.35	25.25±2.87	4.30±0.38	0.60±0.13

Values are mean ± SEM, n = 6.

* $p < 0.05$

Table 3: Effect of Tenofovir-Chitosan Conjugate on Serum Renal Function Parameters.

Treatment	Dose (mg/kg)	Na (mmol/L)	K (mmol/L)	Cl (Mmol/L)	HCO ₃ (Mmol/L)	Urea (Mmol/L)	Creatinine (mg/dL)
Distilled water	5 ml/kg	139.25±1.38	4.15±0.26	100.2±2.14	26.50±1.71	4.30±0.35	0.70±0.09
Tenofovir-chitosan conjugate	10	134.50±2.62	3.50±0.25	93.7±2.78	27.50±1.32	4.55±0.55	0.63±0.09
	100	138.75±3.33	3.43±0.25	92.0±2.35	25.25±2.87	4.30±0.38	0.60±0.13
	1000	133.75±4.71	2.83±0.14*	88.2±0.62*	25.75±2.95	5.28±0.33	0.70±0.15
	1600	139.75±3.33	3.43±0.25	92.0±2.35	25.25±2.87	4.30±0.38	0.60±0.13
	2900	138.75±3.33	3.53±0.25	93.0±2.35	24.25±2.77	4.20±0.28	0.61±0.12
	5000	128.75±3.22	3.44±0.25	92.0±2.35	26.25±2.87	4.30±0.38	0.60±0.23

Values are mean ± SEM, n = 6. * $p < 0.05$

Table 4: Effect of Dolutegravir-Alginate Conjugate on Serum Renal Function Parameters.

Treatment	Dose (mg/kg)	Na (mmol/L)	K (mmol/L)	Cl (Mmol/L)	HCO ₃ (Mmol/L)	Urea (Mmol/L)	Creatinine (mg/dL)
Distilled water	5 ml/kg	139.25±1.38	4.15±0.26	100.2±2.14	26.50±1.71	4.30±0.35	0.70±0.09
Dolutegravir-alginate conjugate	10	133.50±2.62	3.50±0.25	93.7±2.78	27.50±1.32	4.45±0.55	0.63±0.09
	100	138.75±3.33	3.43±0.25	92.0±2.35	25.25±2.87	4.30±0.38	0.60±0.13
	1000	133.75±4.71	2.81±0.13*	88.2±0.61*	25.75±2.95	5.28±0.33	0.70±0.15
	1600	139.76±3.33	3.43±0.25	92.0±2.35	25.25±2.87	4.30±0.38	0.60±0.13
	2900	135.65±3.33	3.53±0.25	93.0±2.35	24.25±2.77	4.20±0.28	0.61±0.12
	5000	129.75±3.22	3.44±0.25	92.0±2.35	26.25±2.87	4.30±0.38	0.60±0.23

Values are mean ± SEM, n = 6. * $p < 0.05$

Table 5: Effect of Efavirenz-Chitosan Conjugates on Haematological Parameters.

Parameter	Control	10 mg/kg	100 mg/kg	1000 mg/kg	1600 Mg/kg	2900 Mg/kg	5000 Mg/kg
RBC ($\times 10^{12}/L$)	1.57 $\pm$ 0.91	0.58 $\pm$ 0.22	1.82 $\pm$ 1.18	3.33 $\pm$ 3.22	0.60 $\pm$ 0.22	2.4 $\pm$ 1.18	4.43 $\pm$ 3.22
HGB (g/dL)	0.68 $\pm$ 0.10	0.50 $\pm$ 0.04	0.35 $\pm$ 0.02	0.44 $\pm$ 0.02	0.50 $\pm$ 0.04	0.35 $\pm$ 0.02	0.46 $\pm$ 0.02
HCT (%)	6.25 $\pm$ 0.83	9.00 $\pm$ 5.50	27.30 $\pm$ 12.24*	1.45 $\pm$ 0.86	9.00 $\pm$ 5.50	27.30 $\pm$ 12.24*	1.45 $\pm$ 0.86
MCHC (g/L)	4.90 $\pm$ 0.73	4.58 $\pm$ 1.29	9.25 $\pm$ 2.81*	6.00 $\pm$ 2.12	4.48 $\pm$ 1.29	9.35 $\pm$ 2.81*	6.00 $\pm$ 2.13
MCH	0.87 $\pm$ 0.16	4.52 $\pm$ 1.46	16.48 $\pm$ 3.37*	0.95 $\pm$ 0.23	5.52 $\pm$ 1.46	18.48 $\pm$ 3.37*	0.95 $\pm$ 0.23
MCV	159.05 $\pm$ 7.45	154.85 $\pm$ 5.65	135.25 $\pm$ 6.81	99.50 $\pm$ 9.90*	154.85 $\pm$ 5.65	135.25 $\pm$ 6.81	99.50 $\pm$ 8.90*
RDW (%)	15.95 $\pm$ 3.12	17.53 $\pm$ 2.34	7.58 $\pm$ 0.71*	13.73 $\pm$ 3.71	18.53 $\pm$ 2.34	7.58 $\pm$ 0.71*	13.73 $\pm$ 3.71
WBC $\times 10^9/L$	17.73 $\pm$ 4.91	10.70 $\pm$ 1.58*	4.84 $\pm$ 1.31*	3.19 $\pm$ 0.28*	10.70 $\pm$ 1.58*	4.84 $\pm$ 1.31*	3.19 $\pm$ 0.28*
LYM (%)	52.08 $\pm$ 1.91	50.38 $\pm$ 0.47	48.73 $\pm$ 1.57	50.25 $\pm$ 1.18	50.38 $\pm$ 0.47	49.73 $\pm$ 1.57	53.25 $\pm$ 1.18
GRA (%)	26.88 $\pm$ 1.13	25.73 $\pm$ 1.17	28.35 $\pm$ 0.67	28.65 $\pm$ 0.58	25.73 $\pm$ 1.17	28.35 $\pm$ 0.67	28.65 $\pm$ 0.58
MID (%)	20.90 $\pm$ 1.03	21.00 $\pm$ 0.18	22.93 $\pm$ 1.44	21.10 $\pm$ 0.63	22.00 $\pm$ 0.18	22.93 $\pm$ 1.44	21.10 $\pm$ 0.63
PLT ($10^9/L$)	268.78 $\pm$ 94.63	2039.80 $\pm$ 70.68*	2229.50 $\pm$ 43.91*	1548.80 $\pm$ 24.85*	2039.80 $\pm$ 70.68*	2229.50 $\pm$ 43.91*	1548.80 $\pm$ 24.85*
PCT (%)	0.20 $\pm$ 0.03	0.73 $\pm$ 0.42	1.00 $\pm$ 0.32	0.49 $\pm$ 0.25	0.71 $\pm$ 0.42	1.00 $\pm$ 0.32	0.49 $\pm$ 0.25
MPV (fL)	7.10 $\pm$ 0.06	6.75 $\pm$ 0.99	6.73 $\pm$ 1.03	6.73 $\pm$ 0.73	6.75 $\pm$ 0.99	6.73 $\pm$ 1.03	6.63 $\pm$ 0.73
PDW (fL)	11.35 $\pm$ 0.09	13.45 $\pm$ 1.94	13.58 $\pm$ 1.08	14.83 $\pm$ 2.09	13.45 $\pm$ 1.94	13.58 $\pm$ 1.08	14.83 $\pm$ 2.09

Values are mean $\pm$ SEM, n = 6. *p<0.05, red blood cell (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), red cell distribution width

(RDW), white blood cells (WBC), lymphocytes (LYM), granulocytes (GRA), MID cells (MID), platelet (PLT) count, plateletcrit (PCT), mean platelet volume (MPV) and platelet distribution width (PDW).

Table 6: Effect of Tenofovir-Chitosan Conjugates on Haematological Parameters.

Parameter	Control	10 mg/kg	100 mg/kg	1000 mg/kg	1600 Mg/kg	2900 Mg/kg	5000 Mg/kg
RBC ($\times 10^{12}/L$)	1.57 $\pm$ 0.91	0.58 $\pm$ 0.22	1.82 $\pm$ 1.18	3.33 $\pm$ 3.22	0.59 $\pm$ 0.22	2.2 $\pm$ 1.18	4.33 $\pm$ 3.22
HGB (g/dL)	0.68 $\pm$ 0.10	0.50 $\pm$ 0.04	0.35 $\pm$ 0.02	0.46 $\pm$ 0.02	0.50 $\pm$ 0.04	0.35 $\pm$ 0.02	0.46 $\pm$ 0.02
HCT (%)	6.25 $\pm$ 0.83	9.00 $\pm$ 5.50	27.30 $\pm$ 12.24*	1.45 $\pm$ 0.86	9.00 $\pm$ 5.50	27.30 $\pm$ 12.24*	1.45 $\pm$ 0.86
MCHC (g/L)	4.90 $\pm$ 0.73	4.58 $\pm$ 1.29	9.25 $\pm$ 2.81*	6.00 $\pm$ 2.12	4.58 $\pm$ 1.29	9.25 $\pm$ 2.81*	6.00 $\pm$ 2.12
MCH	0.87 $\pm$ 0.16	4.52 $\pm$ 1.46	16.48 $\pm$ 3.37*	0.95 $\pm$ 0.23	5.52 $\pm$ 1.46	18.48 $\pm$ 3.37*	0.95 $\pm$ 0.23
MCV	159.05 $\pm$ 7.45	154.85 $\pm$ 5.65	135.25 $\pm$ 6.81	99.50 $\pm$ 9.90*	154.85 $\pm$ 5.65	135.25 $\pm$ 6.81	99.50 $\pm$ 8.90*
RDW (%)	15.95 $\pm$ 3.12	17.53 $\pm$ 2.34	7.58 $\pm$ 0.71*	13.73 $\pm$ 3.71	18.53 $\pm$ 2.34	7.58 $\pm$ 0.71*	13.73 $\pm$ 3.71
WBC $\times 10^9/L$	17.73 $\pm$ 4.91	10.70 $\pm$ 1.58*	4.84 $\pm$ 1.31*	3.19 $\pm$ 0.28*	10.70 $\pm$ 1.58*	4.84 $\pm$ 1.31*	3.19 $\pm$ 0.28*
LYM (%)	52.08 $\pm$ 1.91	50.38 $\pm$ 0.47	48.73 $\pm$ 1.57	50.25 $\pm$ 1.18	50.38 $\pm$ 0.47	49.73 $\pm$ 1.57	53.25 $\pm$ 1.18
GRA (%)	26.88 $\pm$ 1.13	25.73 $\pm$ 1.17	28.35 $\pm$ 0.67	28.65 $\pm$ 0.58	25.73 $\pm$ 1.17	28.35 $\pm$ 0.67	28.65 $\pm$ 0.58
MID (%)	20.90 $\pm$ 1.03	21.00 $\pm$ 0.18	22.93 $\pm$ 1.44	21.10 $\pm$ 0.63	22.00 $\pm$ 0.18	22.93 $\pm$ 1.44	21.10 $\pm$ 0.63
PLT ($10^9/L$)	268.78 $\pm$ 94.63	2039.80 $\pm$ 70.68*	2229.50 $\pm$ 43.91*	1548.80 $\pm$ 24.85*	2039.80 $\pm$ 70.68*	2229.50 $\pm$ 43.91*	1548.80 $\pm$ 24.85*
PCT (%)	0.20 $\pm$ 0.03	0.73 $\pm$ 0.42	1.00 $\pm$ 0.32	0.49 $\pm$ 0.25	0.71 $\pm$ 0.42	1.00 $\pm$ 0.32	0.49 $\pm$ 0.25
MPV (fL)	7.10 $\pm$ 0.06	6.75 $\pm$ 0.99	6.73 $\pm$ 1.03	6.73 $\pm$ 0.73	6.75 $\pm$ 0.99	6.73 $\pm$ 1.03	6.63 $\pm$ 0.73
PDW (fL)	11.35 $\pm$ 0.09	13.45 $\pm$ 1.94	13.58 $\pm$ 1.08	14.83 $\pm$ 2.09	13.45 $\pm$ 1.94	13.58 $\pm$ 1.08	14.83 $\pm$ 2.09

Values are mean $\pm$ SEM, n = 6. *p<0.05, red blood cell (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), red cell distribution width

(RDW), white blood cells (WBC), lymphocytes (LYM), granulocytes (GRA), MID cells (MID), platelet (PLT) count, plateletcrit (PCT), mean platelet volume (MPV) and platelet distribution width (PDW).

Table 7: Effect of Dolutegravir-Alginate Conjugates on Haematological Parameters.

Parameter	Control	10 mg/kg	100 mg/kg	1000 mg/kg	1600 Mg/kg	2900 Mg/kg	5000 Mg/kg
RBC ($\times 10^{12}/L$)	1.57 $\pm$ 0.91	0.58 $\pm$ 0.22	1.83 $\pm$ 1.18	3.33 $\pm$ 4.22	0.58 $\pm$ 0.22	2.2 $\pm$ 1.18	4.45 $\pm$ 3.32
HGB (g/dL)	0.68 $\pm$ 0.10	0.50 $\pm$ 0.04	0.35 $\pm$ 0.02	0.46 $\pm$ 0.02	0.50 $\pm$ 0.04	0.35 $\pm$ 0.02	0.46 $\pm$ 0.02
HCT (%)	6.25 $\pm$ 0.83	9.00 $\pm$ 5.50	27.30 $\pm$ 12.24*	1.45 $\pm$ 0.86	9.00 $\pm$ 5.50	27.30 $\pm$ 12.24*	1.45 $\pm$ 0.86
MCHC (g/L)	4.90 $\pm$ 0.73	4.58 $\pm$ 1.29	9.25 $\pm$ 2.81*	6.00 $\pm$ 2.12	4.58 $\pm$ 1.29	9.25 $\pm$ 2.81*	6.00 $\pm$ 2.13
MCH	0.88 $\pm$ 0.16	4.52 $\pm$ 1.46	16.48 $\pm$ 3.47*	0.95 $\pm$ 0.23	5.52 $\pm$ 1.46	18.4 $\pm$ 3.38*	0.95 $\pm$ 0.23
MCV	159.05 $\pm$ 7.45	154.85 $\pm$ 5.65	135.25 $\pm$ 6.81	99.50 $\pm$ 9.90*	154.85 $\pm$ 5.65	135.25 $\pm$ 6.81	99.51 $\pm$ 8.90*
RDW (%)	15.96 $\pm$ 3.12	17.53 $\pm$ 2.34	7.58 $\pm$ 0.71*	13.73 $\pm$ 3.71	18.53 $\pm$ 2.34	7.58 $\pm$ 0.71*	13.73 $\pm$ 3.71
WBC $\times 10^9/L$	17.73 $\pm$ 4.91	10.70 $\pm$ 1.58*	4.84 $\pm$ 1.32*	3.19 $\pm$ 0.28*	10.70 $\pm$ 1.58*	4.84 $\pm$ 1.31*	3.19 $\pm$ 0.28*
LYM (%)	52.08 $\pm$ 1.91	50.38 $\pm$ 0.47	48.73 $\pm$ 1.57	50.25 $\pm$ 1.18	50.38 $\pm$ 0.47	49.73 $\pm$ 1.57	53.25 $\pm$ 1.18
GRA (%)	26.88 $\pm$ 1.13	25.73 $\pm$ 1.17	28.35 $\pm$ 0.67	28.65 $\pm$ 0.58	25.73 $\pm$ 1.17	28.35 $\pm$ 0.67	28.65 $\pm$ 0.58

MID (%)	20.90±1.03	21.00±0.18	22.93±1.44	21.10±0.63	22.00±0.18	22.93±1.44	21.10±0.63
PLT (10 ⁹ L)	268.78±94.63	2039.80±70.68*	2229.50±43.91*	1548.80±24.85*	2039.80±70.68*	2229.50±43.91*	1548.80±24.85*
PCT (%)	0.20±0.03	0.73±0.42	1.00±0.32	0.49±0.25	0.71±0.42	1.00±0.32	0.49±0.25
MPV (fL)	7.10±0.06	6.75±0.99	6.73±1.03	6.73±0.73	6.75±0.99	6.73±1.03	6.63±0.73
PDW (fL)	11.35±0.09	13.45±1.94	13.58±1.08	14.83±2.09	13.45±1.94	13.58±1.08	14.83±2.09

Values are mean ± SEM, n = 6. *p<0.05, red blood cell (RBC), hemoglobin concentration (HBG), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), red cell distribution width

(RDW), white blood cells (WBC), lymphocytes (LYM), granulocytes (GRA), MID cells (MID), platelet (PLT) count, platelet crit (PCT), mean platelet volume (MPV) and platelet distribution width (PDW).

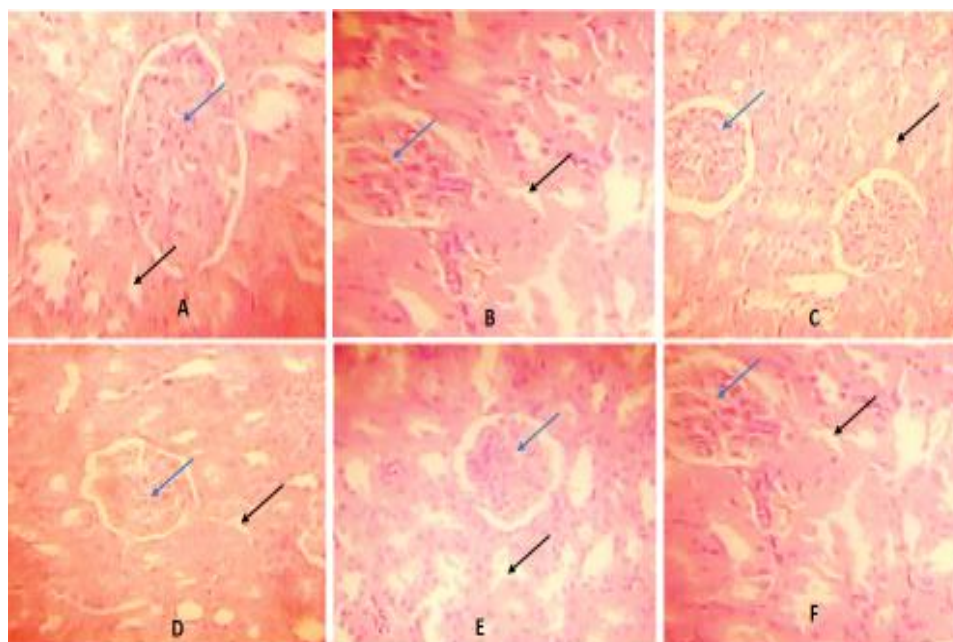


Plate 1: Photomicrograph of the Kidney of Wistar rats treated with Efavirenz-chitosan, Tenofovir-chitosan and Dolutegravir-alginate.

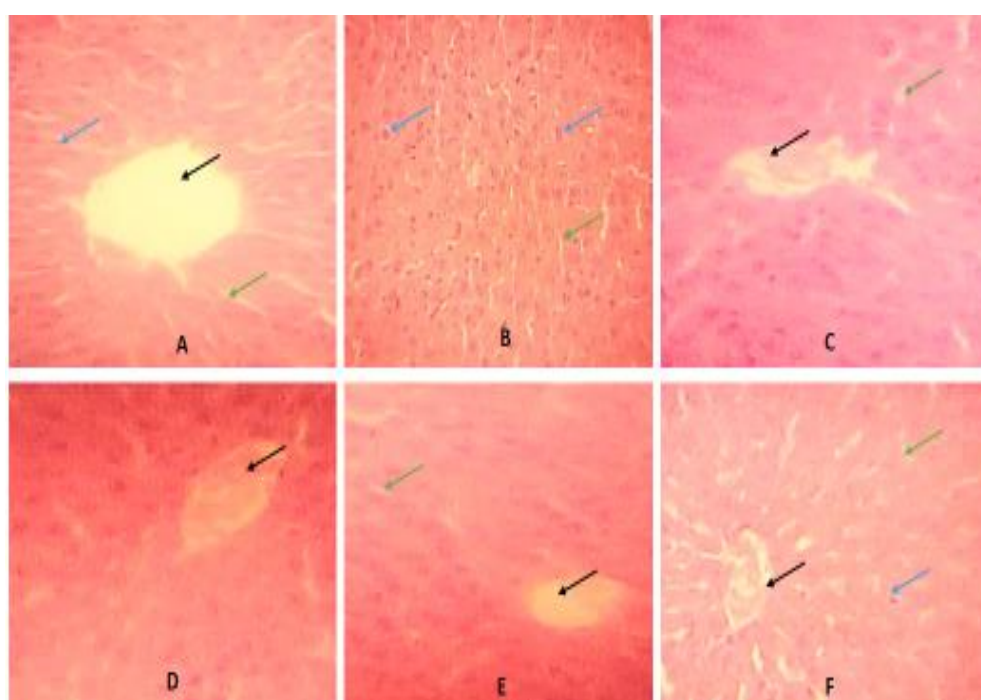


Plate 2: Photomicrograph of the livers of Wistar rats treated with Efavirenz- chitosan, Tenofovir-chitosan and Dolutegravir-alginate.

Table 8: Preliminary Screening of Selected Antiretroviral drugs.

Conc. (µg/ml)	Mean trypanosome count/field				
	Dolutegravir	Lopinavir	Tenofovir	Efavirenz	Diminazine
500	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
125	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
62.5	0.00±0.00	0.33±0.58	0.00±0.00	0.00±0.00	0.00±0.00
31.25	0.00±0.00	1.33±0.57	1.33±1.53	0.00±0.00	0.33±0.58
15.625	0.00±0.00	1.67±1.53	6.33±2.08	0.00±0.00	1.00±1.15
7.8125	0.33±0.58	1.00±0.00	8.00±1.73	0.00±0.00	1.67±1.15
3.90625	1.00±0.00	1.00±0.00	9.33±1.55	0.00±0.00	2.33±0.01
1.953125	1.67±0.58	3.33±0.51	10.3±1.53	0.00±0.00	2.33±0.08
0.9765625	2.33±1.15	6.31±0.49	10.3±1.53	0.00±0.00	2.67±1.15
0.48828125	5.33±0.58	6.67±0.57	10.3±0.58	0.00±0.00	3.67±0.58
0.244140625	6.36±1.52	6.67±1.15	10.7±2.08	0.00±0.00	5.33±1.53
0.1220703125	5.67±0.58	8.67±2.08	12.7±1.53	1.67±1.13	6.00±1.00
0.06103515625	7.33±1.15	10.0±2.00	13.3±1.54	6.00±2.00	6.33±0.57
0.030517578125	9.67±1.15	10.7±1.53	14.7±1.15	5.67±1.53	6.67±1.15
0.0152587890625	11.7±1.15	11.0±1.73	15.3±1.52	7.33±0.57	9.00±1.73
0.00762939453125	13.3±2.52	15.7±1.15	15.7±0.57	8.33±0.58	9.67±2.08

Values are mean ± SEM, n = 3. $p < 0.01$

Table 9: Antitrypanosoma Screening of Drug Conjugates.

Conc. (mg/ml)	Mean Trypanosome Counts/Field				
	Dolutegravir-Alginate	Tenofovir-chitosan	Efavirenz-chitosan	Distill water Negative control	Diminazine aceturate Positive control
10.0	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	21.7±1.20 ^e	0.00±0.00 ^a
5.0	0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a	21.7±1.20 ^e	0.00±0.00 ^a
2.5	0.00±0.00 ^a	11.0±1.15 ^b	0.00±0.00 ^a	21.7±1.20 ^e	0.00±0.00 ^a
1.25	2.67±0.67 ^b	12.0±1.73 ^{bc}	0.00±0.00 ^a	21.7±1.20 ^e	0.00±0.00 ^a
0.625	4.33±0.86 ^b	15.0±1.00 ^{cd}	0.00±0.00 ^a	21.7±1.20 ^e	0.00±0.00 ^a
0.3125	8.67±0.88 ^c	15.3±2.52 ^d	0.00±0.00 ^a	21.7±1.20 ^e	1.00±0.00 ^a
0.15625	9.67±0.88 ^c	17.2±1.73 ^d	0.00±0.00 ^a	21.7±1.20 ^e	2.33±0.33 ^b
p value	< 0.001	< 0.001	< 0.001	< 0.001	

Values are given as mean ± standard deviation. In each column, values with different superscripts have

statistically significant. $p < 0.01$ (a,b&c are significant while d&e are not significant).

Table 10: Antitrypanosoma Screening of Drug Conjugates showing their minimum inhibitor concentration (MIC) and confidence interval (CI).

Sample	MIC (µg/ml)	LC ₅₀ (mg/ml)	95% CI of LC ₅₀ (mg/ml)	Regression equation
Tenofovir-Chitosan	5.0	0.951	0.297-2.695	$Y = 0.932 + 1.447x$
Efavirenz-Chitosan	0.15625	0.814	0.614-1.076	$Y = 0.997 + 2.208x$
Dolutegravir-Alginate	2.5	0.177	0.078-0.276	$Y = 0.9711 + 1.610x$
Distill water (negative control)	> 100	1.090	2.099-14.817	$Y = -0.458 + 0.749x$
Diminazine (positive control)	0.625	0.05	-	$Y = 2.914 + 2.240x$

LC₅₀: Median lethal concentration, MIC: Minimum inhibitory concentration, CI: Confidence interval. $Y = Mx + C$, Y = Dose, x = Mortality.

4.0 DISCUSSION

4.1 TOXICOLOGY STUDY OF THE CONJUGATES

Toxicity profile of any synthesised drug is evaluated to find and establish adverse effect, its significance and exposure level at which this effect is observed. LD₅₀ is used to determine the safety or toxicity of a substance.^[8]

Our results from the acute toxicity study revealed that the drug conjugates (efavirenz-chitosan, tenofovir-chitosan and dolutegravir-alginate) administered via oral route to the Wistar rats at 5000mg/kg following Lork's method of testing acute toxicity failed to produce any sign of toxicity and/or mortality. Therefore, the LD₅₀ was greater than 5000 mg/kg and thus classified as relatively safe.^[7,8] Previous investigation by Jenna et al.,^[6] on the LD₅₀ of efavirenz and tenofovir revealed toxicity. The deviation from the current finding may be attributed to the fact that

the drugs are conjugated to polymers which upon enzymatic action releases the parent drug slowly.

Substances that are dosed frequently in the course of disease treatment may require repeated dosing toxicological evaluation (sub-chronic study). This is because daily use may amount to drug accumulation in the body leading to impairment of tissues and organs.^[7,8] Therefore, sub-chronic toxicity testing is essential in assessing the impact of the substance on the target organ, hematological or biochemical parameters which are not assessed in acute toxicity study.^[7,8] Observable marks of toxicity or mortality in the rats' functional characteristics of blood and blood forming organs such as the liver and kidney.^[6,7,8] The extend of toxicity of the conjugates are often determined through assessment of these parameters. Hematological parameters are useful indices that can be employed to access the toxic potentials of the synthesised product in the living system.^[6] They can be used to explain blood relating functions of chemical compound.^[9] The haemoglobin concentration and hematocrit are values revealing the degree of anemia while the MCHC is a useful index of the average hemoglobin concentration of the red cells.^[5]

Generally, low readings for RBC, Hb and hematocrit indicates anemia. At 200% 400mg/kg dose, all parameters studied were not significantly affected by all the conjugates synthesised (efavirenz-chitosan, Tenofovir-chitosan and dolutegravir-alginate) compared to the control group. Significant decrease in RBC, HGB, PLT and MCV at 100mg/kg dose level indicate that the conjugates interfere with the normal production of haemoglobin and its concentration within RBC and may thus possess the potential to cause anemia at this dose level.^[8] In addition, the significant ($p < 0.05$) decrease in the hemoglobin and hematocrit levels at 100mg/kg body weight dose could be the optimal concentration of the product which may cause effect on the red blood cells indices. Efavirenz alone have been found to be cytotoxic and cytolytic and can produce anemia Joseph *et al.*,^[5] therefore low red cells indices including hematocrit and hemoglobin observed may be attributed to the presence of efavirenz in the efavirenz-chitosan conjugate. Also, tenofovir has the ability to lower serum hemoglobin levels therefore, the decrease in hemoglobin level experience during the hematological test is probably due to the presence of tenofovir in the tenofovir-chitosan conjugate.^[6]

Dolutegravir as an antiretroviral drug has a good tolerability history therefore has no remarkable haematological indices compared to that of efavirenz and tenofovir in their respective conjugates. Chemicals produce a wide variety of clinical and pathological hepatic injury, biochemical markers (e.g. alanine transferase, alkaline phosphatase and bilirubin) are often used to indicate liver damage.^[10] Liver injury is defined as a rise in either (a) ALT level more than three times of upper limit of normal (ULN), (b) ALP level more than

twice ULN or (c) total bilirubin level more than twice ULN when associated with increased ALT or ALP.^[11] Liver damage is further characterised into hepatocellular (predominantly initial alkaline transferase elevation) and cholestatic (initial alkaline phosphate rise) types. However, they are not mutually exclusive and mixed types of injuries are often encountered.^[5] The biochemical indices monitored in the liver is a useful marker for assessment of tissue damage. The measurement of activities of various enzymes in the tissues and body fluids plays a significant role in disease investigation and diagnosis.^[12] Tissue enzymes can also indicate tissue cellular damage caused by chemical compounds, long before structural histological techniques.^[12]

Alkaline phosphate, a marker enzyme for plasma and endoplasmic reticulum,^[6] is often employed to assess the integrity of plasma membrane.^[5] In this study, that is antiretroviral conjugate of polymers (efavirenz-chitosan, tenofovir-chitosan and dolutegravir-alginate conjugate) did not cause significant change in most of liver function test values. This indicate that though some of the antiretroviral drugs in the polymer conjugates synthesised like the efavirenz and tenofovir have some toxicological issues, but did not manifest excessively in the conjugate probably due to its conjugation with the polymers which eventually masked the toxic parameter of the drugs and its slow-release nature from the conjugate.^[13]

The result of both the haematological and histological test of the conjugates synthesised was far better compared to the studies by,^[6] for both efavirenz and tenofovir respectively, where haematological and histological parameters were studied. Dolutegravir-alginate conjugate has a better histological and hematological profile in the cause of the study, this is in accordance with Brown & Fabro,^[14] where no toxicity was observed with dolutegravir in rat whole embryo culture, making it suitable for HIV therapy. It was not surprising that even the dolutegravir- alginate conjugate gave a good toxicological profile when compared to the other conjugates (efavirenz-chitosan and Tenofovir-chitosan).

4.2 BIOLOGICAL EVALUATION OF DRUGS ALONE AND DRUG CONJUGATES

4.2.1 Pilot Study/ Screening of Antiretroviral Drugs

The antiretroviral drugs that were screened in the pilot study against the Trypanosoma showed significant activity, with efavirenz having the highest activity from the concentration of 0.2441µg/ml to 500 µg/ml and minimum concentration (MIC) of 0.12207 µg/ml. Followed by Dolutegravir with MIC of 7.8175 µg/ml, then tenofovir and lopinavir with MIC of 62.5 µg/ml while diminazens (positive control) had a MIC of 31.25 µg/ml.

The pilot study of these antiretroviral drugs screened against the *Trypanosoma* spp confirmed its activity as earlier reported by.^[15] The pilot study and the claim by Zhan et al,^[15] informed the choice of these antiretrovirals (efavirenz, tenofovir and dolutegravir) that were conjugated to the polymers (chitosan and alginate) to tackle the challenge of toxicity and resistance that the traditional antitrypanosoma and efavirenz possessed.

4.2.2 Antitrypanosoma Drug Conjugate Evaluation

The antitrypanosoma activity of efavirenz-chitosan, tenofovir-chitosan and dolutegravir-alginate complex and its solution against a 7-day *Trypanosoma brucei* using suppressive test in rat gave better activity when compared with the initial pilot screening of the antiretroviral drug alone. When the drug conjugates were evaluated against the *Trypanosoma brucei*, efavirenz-chitosan conjugate has a better activity than diminazen which serves as the positive control drug with a confidence interval (IC₅₀) value of 28% better than efavirenz alone. The minimum inhibitory concentration (MIC) of the efavirenz-chitosan is 0.15625 µg/ml while that of efavirenz alone is 0.244 µg/ml. This increase in MIC of the conjugate might be due to the synergistic effect of chitosan and also the sustained release of the efavirenz from the conjugate.^[16] For tenofovir-chitosan conjugate, the antitrypanosoma activity is far better than the parent drug (tenofovir) alone with IC₅₀ value of 32% and MIC of 5.0 µg/ml while that of tenofovir alone is 62.5 µg/ml, the wide difference in this *Trypanosoma* activity might be due to the fact the chitosan which serves as the biodegradable polymer has a synergistic effect with the tenofovir and the fact that the drug alone (tenofovir) could be release in sustained quantity after being acted upon by hydrogenase enzyme makes it highly active when compared to tenofovir alone.^[17]

Dolutegravir-alginate conjugate evaluated against *Trypanosoma brucei* was found to possess a better activity against both the control (diminazen) and the drug alone (dolutegravir) with IC₅₀ and MIC of 52% and 15.625 µg/ml (Fig. 11) while that of dolutegravir-alginate is 2.5 µg/ml (Fig. 12). The same explanation for the reason of increased activity in the previous conjugates applies to dolutegravir-alginate conjugate. The conjugate is an ester product that could be acted upon by esterase enzymes releasing the parent drug (dolutegravir) at a slow and sustained release from its conjugate bringing about the slight increase in anti-*Trypanosoma* activity. Comparatively, the anti-*Trypanosoma* activity of the conjugate in the present study is better than that obtained for a closely related synthesis in a previous study,^[17] where synthesis of tetrahydroisoquinoline derivatives was evaluated against *Trypanosoma brucei rhodesiense* with MIC (0.25–70.5 µg/ml). In another study,^[18] where it involves using human and murine cell models which demonstrated that HIV-IPs extensively affect the mitochondrion and the endoplasmic reticulum of the

Trypanosoma brucei, shows that the present study has better anti-*Trypanosoma* activity.

5.0 CONCLUSION

In this study, the LD₅₀ of the conjugates were found to be greater than 2000 mg/kg which provided initial evidence of the potential safety of the conjugates. Likewise, there was neither mortality or observable adverse effects recorded during the period of the study.

The biological evaluation of the three antiretroviral drugs and drug-polymer conjugates against the *Trypanosoma brucei*, shows considerable activity with the conjugates having better antitrypanosoma activity than the drug alone. Efavirenz-chitosan conjugate, tenofovir-chitosan and dolutegravir-alginate have better activity than the positive control.

In conclusion, the present study showed that the conjugates of dolutegravir, efavirenz and tenofovir may be considered as comparatively safe of toxicity as none of the conjugates produced any haematological challenge and no distortion of the architecture of the tissue. The study corroborated the application of the drug conjugates as better antitrypanosomal agent with lowest toxicity effect when compared with the melarsoprol, eflornithine, pentamidine and suramin.

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