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Biological safety of leech salivary extract-mediated silver nanoparticles in wistar rats

Babayi, H.[1,2*], Owolabi, B. I.[1], Amali, E. D.[3], Aboyeji, D. O.[1], Isah, R. M.[1,4]

[1]Department of Microbiology, School of Life Sciences, Federal University of Technology, P.M.B. 65, Minna, Niger State, Nigeria
[2]Centre for Genetic Engineering and Biotechnology, Federal University of Technology, P.M.B. 65, Minna, Niger State, Nigeria
[3]Department of Pharmaceutical Microbiology, University of Ibadan, Ibadan, Oyo State, Nigeria
[4]Department of Microbiology, Ibrahim Badamasi Babangida University, PMB 11 Lapai, Niger State, Nigeria

[1,2*]Corresponding Author's Email: acadbabayi@yahoo.com

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ABSTRACT

Leeches are annelid worms that feed on blood and dwell in freshwater environments. Both leech saliva and silver nanoparticles have been shown to possess remarkable antibacterial properties against microorganisms. Although the worms are considered dangerous, their saliva may not be harmful. Silver nanoparticles mediated by leech salivary extract (LSE-Ag) were biologically synthesized and studied using UV spectroscopy and a nanosizer. Using standard method, the acute (LD_{50}) and sub-acute toxicity of LSE-Ag were evaluated. The LSE-Ag had a wavelength of 456 nm and a size of 98.04 nm. The LSE-Ag LD_{50} in rats was above 5000 mg/kgbw. Oral delivery of LSE-Ag to rats (25, 50 and 100 mg/kgbw) for 21 days showed no significant ($P > 0.05$) change in body weight, differential blood count (lymphocytes, monocytes, neutrophils, eosinophils, and basophils), red blood cell indices (Platelets, HB, WBC, RBC, PCV, MCV, MCH and MCHC), renal function indices (urea, sodium, creatinine and potassium) and liver biomarkers (protein, albumin, alkaline phosphatise, alanine amino transferase, and aspartate amino transferase). When compared to the control, LSE-Ag at 100 mg/kgbw increased absolute spleen weight and decreased feed consumption in treated rats. The findings show that LSE-Ag is relatively safe and could be a rich source of antimicrobial agent but caution should be taken at higher doses.

Keywords: LSE-Ag, Acute toxicity, Sub-acute toxicity, Liver biomarkers, RBC indices.

INTRODUCTION

Leeches are therapeutic flatworms that feed on the blood of vertebrates and were once used in the practice of bloodletting (Alharbi, 2015; Kamath, 2020). Their body is segmented with both posterior and anterior suckers (Faleh *et al.,* 2019; Kuo and Lai, 2019). They are members of the "*Annelida*" phylum, the "*Arhynchobdellida*" order, and the "*Hirudinidae*" family. *Hirudo medicinalis, Hirudo verbena* and *Hirudo orientalis* are among the fifteen medicinal species identified (Wollina *et al.,* 2016) of about six hundred (600) species in total. A variety of substances, including antimicrobial peptides (AMP), proteins, and peptides (Abdualkader *et al.,* 2011), have been discovered in the salivary secretions of some species of Leeches (Ghawi *et al.,* 2012; Ojo *et al.,* 2018). These substances have been shown to suppress microorganism growth, alleviate hypoglycemia, and have potential to reduce silver ions (Ag^+) in the nanoparticles synthesis (Ahmad *et al.,* 2019). Nanoparticles (NPs) are small particles having a diameter ranging from 1 to 100 nanometers (Christian *et al.,* 2008; Pal *et al.,* 2011; Khan *et al.,* 2019). They are often synthesized from transition metals in the periodic table using a variety of chemical, physical, biological, or biogenic approaches (Patra and Baek, 2014; Godwin *et al.,* 2015). They have a wide range of applications in diverse areas such as drug delivery (De Jong and Borm, 2008), antimicrobial activities, bio-sensing, imaging, bioremediation of some contaminant, and water treatment, to name a few (Tiquia-Arashiro and Rodrigues, 2017; Sajid and Plotka-Wasylka, 2020). Organic chemicals found in natural products such as plant or animal extracts, as well as microbial secretions,

can act as natural or biogenic capping and stabilizing agents, decreasing metallic particles to nanoparticles (Nadarolu *et al.,* 2017; Singh *et al.,* 2018; Zhang *et al.,* 2020). Extracts of plants, animals, and even biologically synthesized nanoparticles have successfully been utilized in the quest of finding an effective alternative to antimicrobial agents that are being circumvented by resistant superbugs (Lee *et al.,* 2019; Benitez-Chao *et al.,* 2021; McNeilly *et al.,* 2021).

Antimicrobial agents are medications, chemicals, or other substances derived from animal and plant products, microbes, or chemically produced substances that can inhibit, impede, or destroy the growth of bacteria (Parasuraman, 2011). A good antimicrobial agent should be able to inhibit or destroy microbe development while remaining safe for human consumption. Toxicology research is used to evaluate and confirm the safety of medications, foods, and antimicrobial agents (Parasuraman, 2011). Toxicological investigations use a variety of techniques to profile or characterize the effects of medicines and antimicrobial agents on organ structure and function (Arome and Chinedu, 2013; BioAgilytix lab, 2021). This study aimed to evaluate effects of LSE-Ag on serum and haematological parameters in wistar rats.

MATERIALS AND METHODS

Leech collection and identification

In the month of June 2019, leeches were collected using a scooping net along the shorelines of a fresh water dam in Bosso, Niger State, and Panda Development Area, Nassarawa State, Nigeria. Dr. Azubuike U. C. of the Department of Plant Biology, Federal University of Technology, Minna, Nigeria, identified the worms as *Hirudo medicinalis*.

Leech Maintenance in the Laboratory

The leeches were housed at room temperature (25 ± 2 °C) in well-aerated plastic containers filled with non-chlorinated water (borehole water) throughout the research. The water was changed every two days after the leeches' saliva was extracted, and the leeches were fed cow blood every three weeks (Abdualkader *et al.,* 2014).

Leech Saliva Extraction

The ice-shocking approach was used to obtain leech saliva, as explained by Abdualkader *et al.* (2014) and Ojo *et al.* (2018). In a test tube was placed two to three pieces of leeches which was inserted in a bowl filled with ice blocks for 20 minutes and the leeches regurgitated their intestinal contents into the sterile test tube after full paralysis. Saliva was aspirated using a sterile hypodermic needle and syringe, then transferred to sterile screw-capped containers and stored at - 4 °C.

Experimental Model

Experimental animals

The acute and subacute toxicity investigations were conducted on twenty-seven (27) Wister rats (*Rattus novergicus*) of 8 weeks old and average weight of 150-200g. The rats were obtained in Jos, Plateau State, Nigeria, and were given two weeks to acclimate. The rats were kept in plastic cages bedded with clean dried saw dust (wood shavings), appropriately aerated and kept in a well separated and ventilated housing. The rats were fed *ad libitum* with regular animal diets and tap water. On a daily basis, sanitation and the replacement of soiled wood shavings were observed (Babayi *et al.,* 2018). The body weight, feed, and water intake were all accurately monitored and recorded. The animals were housed and cared for in accordance with Good Laboratory Practice (GLP) regulations of WHO (1998). The principles of Laboratory Animal Care (1985) were also followed throughout the study.

Toxicological investigations on silver nanoparticles mediated by leech salivary extract

The acute and sub-chronic toxicity investigations of leech salivary extract-mediated silver nanoparticles was carried out as described by Shittu *et al.* (2015). The research involved determining the LD_{50} and assessing the long-term effects of low nanoparticle concentrations on haematological markers and serum biochemical indices in rats.

Acute toxicity investigations of silver nanoparticles mediated by leech salivary extract

The LD_{50} of the leech salivary extract-mediated silver nanoparticles (LSE-Ag) was determined using the method of Lorke (1983) in order to establish a tolerable dose range for rats. The study was carried out in three phases. In the first phase, nine rats randomly divided into three groups of three rats each were given 10, 100 and 1000 mg/kg body weight of extract orally (via a cannula), respectively. The rats were observed for signs of adverse effects and death for 24 hours. In the second phase of the study, the procedure was repeated using another fresh set of three groups of two rats each given 1500, 2000 and 2500 mg/kg body weight of extract respectively. The rats were also observed for signs of toxicity, mortality and weighed for 14 days. Based on results from the second phase, 3000 mg/kgbw and 5000 mg/kgbw of the extract were administered to another fresh set of two groups of one rat each in the third phase. The rats were observed for signs of toxicity and mortality

for the first 4 hours and thereafter daily for 14 days. The volume of LSE-Ag to be administered was calculated using the following equation in relation to the weight of the rats:

$$\text{Volume (mL)} = \frac{\text{Dose to administer X Weight of animal (g)}}{\text{Concentration (mg/mL)}} X \frac{1000\ g}{1}$$

Determination of sub-acute toxicity of silver nanoparticles mediated by leech salivary extract

The method of Aniagu *et al.* (2005) was utilized to determine the sub-chronic toxicity of LSE-Ag. Fifteen rats were chosen by stratified randomization and divided into five groups of three rats each. The principal treatment of LSE-Ag was given to groups one through three at concentrations of 25, 50 and 100 mg/kgbw, respectively. Groups four and five received 0.2 ml of normal saline as a normal control and 0.2 ml of 2 mM silver nitrate ($AgNO_3$) solution as a positive control, respectively daily for 28 days. On a daily basis, the body weight, feed, and water intake of the rats were measured once daily over the 28 days period. At the end of the twenty-eight-day treatment, the rats were starved overnight before being slaughtered under chloroform anesthesia. Blood was obtained in heparin bottles and plain bottles for the haematology and serum biochemistry testing, respectively (Shittu *et al.,* 2015). Each rat's liver, heart, kidneys, lungs, and spleen were removed and weighed (Srinivasan, 2018).

Statistical Analysis

The data in this study was expressed using mean value ± standard error of the mean (S.E.M.) Comparisons between groups were made using Analysis of Variance (ANOVA). Using the Statistical Package for Social Sciences (SPSS) version 21, the Duncan Multiple Range Test (DMRT) was performed to assess significant differences between the control and experimental groups.

RESULTS

Characteristics of leech salivary extract-mediated silver nanoparticles

Leech salivary extract-mediated silver nanoparticles formed was a dark brown precipitate. The wavelength of the precipitate was at 456 nm and the absorbance was 0.37 (Fig. 1). The size of the particle was 98 nm (Fig. 2).

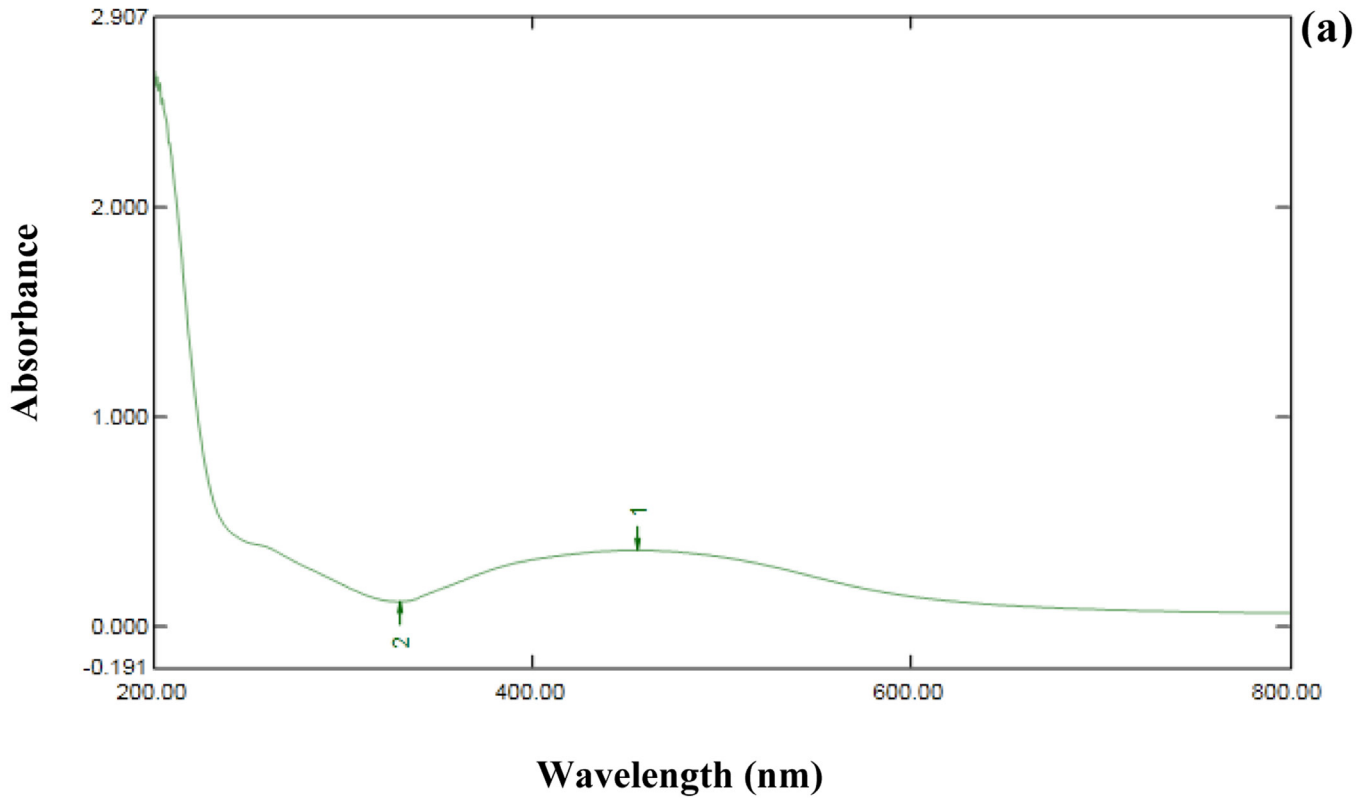

Figure 1: Ultraviolet-visible-spectrum of leech salivary extract-mediated silver nanoparticles

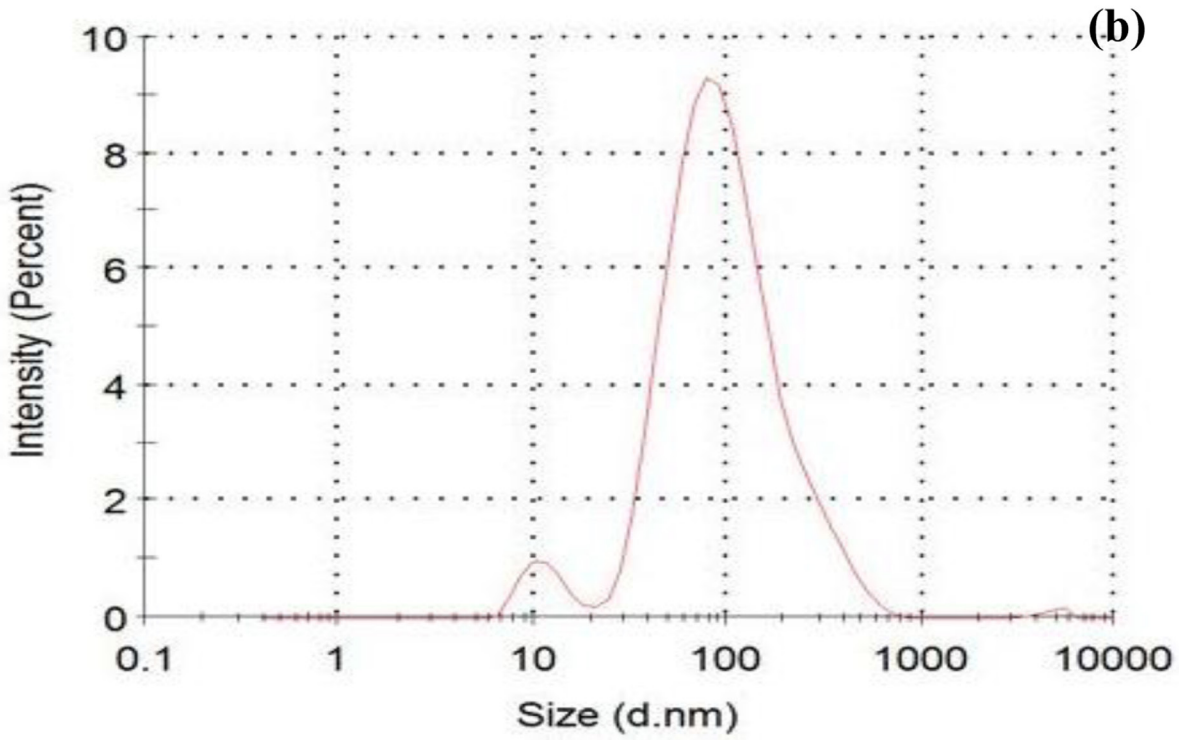

Figure 2: Zeta nano size distribution pattern of leech salivary extract-mediated silver nanoparticles

Acute toxic dose of leech salivary extract-mediated silver nanoparticles in wistar albino rats

Table 1 shows the acute toxic dose (LD_{50}) of Leech salivary extract-mediated silver nanoparticles in Wister albino rats. The administrations of 100 to 5000 mg/kgbw of the LSE-Ag were not accompanied by physical and behavioural changes. The conjugate's LD_{50} is higher than 5000 mg/kgbw.

Table 1: Acute toxic dose of leech salivary extract-mediated silver nanoparticles in wistar albino rats.

Number of rats	Doses (mg/kgbw)	Mortality	Toxicity signs
3	10	0	NS
3	100	0	NS
3	1000	0	NS
2	1500	0	NS
2	2000	0	NS
2	2500	0	NS
1	3000	0	NS
1	5000	0	NS

mg/kgbw: milligram per kilogram body weight; NS: No toxicity sign

Effect of silver nanoparticles mediated by leech salivary extract on the body weight of wistar albino rats

The effect of silver nanoparticles mediated by leech salivary extract on wistar albino rats' body weight is shown in Table 2. The body weights of rats in all three groups (25, 50 and 100 mg/kgbw) were not statistically different ($P > 0.05$) from the control groups (rats treated with $AgNO_3$ and normal saline).

Table 2: Effect of silver nanoparticles mediated by leech salivary extract on the body weight of wistar albino rats.

Treatment (mg/kgbw)	Time (weeks)			
	0	1	2	3
25	148.77 ± 15.63[a]	159.62 ± 17.62[a]	161.89 ± 16.89[a]	177.91 ± 16.81[a]
50	157.02 ± 8.54[a]	148.82 ± 6.96[a]	152.07 ± 5.47[a]	160.56 ± 6.14[a]
100	161.23 ± 13.094[a]	172.91 ± 20.48[a]	190.17 ± 42.21[a]	161.14 ± 9.44[a]
Ns	150.87 ± 13.99[a]	161.83 ± 18.21[a]	167.29 ± 17.77[a]	181.62 ± 12.39[a]
$AgNO_3$	132.20 ± 10.63[a]	146.84 ± 6.03[a]	154.41 ± 4.05[a]	162.99 ± 7.95[a]

Values are expressed as means ± SEM. There is no significant difference (P > 0.05) between values in a column with the same superscript. Ns: Normal saline (normal control), $AgNO_3$: Silver nitrate (positive control), mg/kgbw: milligram per kilogram body weight

Effects of silver nanoparticles mediated by leech salivary extract on the absolute organ weight of wistar albino rats

Table 3 shows the effect of silver nanoparticles mediated by leech salivary extract on wistar albino rats' absolute organ weight. The results showed that the weights of the rats' heart, lungs, kidneys, and liver were not significantly different (P > 0.05) from the control groups (rats treated with $AgNO_3$ and normal saline) in any of the treated groups (25, 50 and 100 mg/kgbw). However, the spleen of the rats exposed to 100 mg/kgbw was significantly (P < 0.05) increased when compared to the treated and control groups.

Table 3: Effect of silver nanoparticles mediated by leech salivary extract on the absolute organ weight of wistar albino rats.

Treatment (mg/kgbw)	Absolute Organ weight (g)				
	Heart	Spleen	Lungs	Kidney	Liver
25	0.35 ± 0.00[a]	0.16 ± 0.02[a]	0.77 ± 0.01[a]	0.53 ± 0.01[a]	3.60 ± 0.51[a]
50	0.27 ± 0.06[a]	0.19 ± 0.03[a]	0.93 ± 0.28[a]	0.68 ± 0.06[a]	3.77 ± 0.33[a]
100	0.45 ± 0.11[a]	0.36 ± 0.03[b]	0.88 ± 0.12[a]	0.81 ± 0.18[a]	4.85 ± 0.62[a]
Ns	0.32 ± 0.01[a]	0.22 ± 0.01[a]	0.76 ± 0.02[a]	0.55 ± 0.02[a]	3.71 ± 0.62[a]
$AgNO_3$	0.41 ± 0.02[a]	0.20 ± 0.02[a]	0.73 ± 0.01[a]	0.61 ± 0.03[a]	4.01 ± 0.07[a]

Values are expressed as means ± SEM. There is no significant difference (P > 0.05) between values in a column with the same superscript. g: grams, Ns: Normal saline (normal control), $AgNO_3$: Silver nitrate (positive control)

Effect of silver nanoparticles mediated by leech salivary extract on feed intake of wistar albino rats

The effect of silver nanoparticles mediated by leech salivary extract on the feed intake of Wistar albino rats is displayed in Table 4. At week 1, there was no significant difference (P > 0.05) between any of the treatment groups, while at week 2; there was no significant difference between groups 1, 3, and 5. However, the animals given normal saline (normal control) consumed the most feed, followed by those given 100 mg/kgbw of LSE-Ag, and those given 50 mg/kgbw of LSE-Ag consumed the least. There was no significant difference (P > 0.05) between the treatment groups at weeks 1 and 3.

Table 4: Effect of silver nanoparticles mediated by leech salivary extract on feed intake of wistar albino rats.

Treatment (mg/kgbw)	Time (weeks)		
	1	2	3
25	49.66 ± 2.84[a]	44.66 ± 7.26[ab]	45.89 ± 6.27[a]
50	44.99 ± 6.82[a]	40.29 ± 11.12[a]	51.15 ± 8.03[a]
100	41.24 ± 5.94[a]	50.92 ± 6.91[ab]	36.00 ± 5.74[a]
Ns	49.77 ± 3.45[a]	67.30 ± 8.45[b]	50.05 ± 4.04[a]
$AgNO_3$	44.96 ± 1.39[a]	53.23 ± 5.39[ab]	50.02 ± 5.83[a]

Values are expressed as means ± SEM. There is no significant difference (P > 0.05) between values in a column with the same superscript. g: grams, Ns: Normal saline (normal control), $AgNO_3$: Silver nitrate (positive control)

Effect of silver nanoparticles mediated by leech salivary extract on the fluid intake of wistar albino rats

The effect of leech salivary extract-mediated silver nanoparticles on wistar albino rats' fluid intake is seen in Table 5. At week 1, the rats administered silver nitrate had the least fluid intake when compared with other groups. Similar fluid intake was observed between groups administered 25 and 50 mg/kgbw of the LSE-Ag. In week 2, groups administered 25, 50 mg/kgbw and normal saline had similar fluid intake while groups administered 100 mg/kgbw and $AgNO_3$ equally had similar fluid intake. In week 3, rats administered with 100 mg/kgbw had the least fluid intake when compared with other groups while groups administered with 50 mg/kgbw and normal saline had similar fluid intake. The group administered with 25 mg/kgbw had the highest fluid intake.

Table 5: Effect of silver nanoparticles mediated by leech salivary extract on fluid intake of wistar albino rats.

Treatment (mg/kgbw)	Time (weeks)		
	1	2	3
25	285.71 ± 17.70[c]	328.33 ± 18.15[b]	322.85 ± 9.68[d]
50	267.14 ± 15.54[c]	300.00 ± 0.00[b]	278.51 ± 12.03[c]
100	180.00 ± 9.51[ab]	188.33 ± 14.00[a]	185.71 ± 13.42[a]
Ns	218.57 ± 10.10[b]	283.33 ± 23.47[b]	275.71 ± 9.47[c]
$AgNO_3$	165.71 ± 18.63[a]	218.33 ± 10.13[a]	245.71 ± 4.28[b]

Values are expressed as means ± SD. There is no significant difference (P > 0.05) between values with the same superscript. Ns: Normal saline (normal control), $AgNO_3$: Silver nitrate (positive control), mg/kgbw: milligram per kilogram body weight, SD: standard deviation

Effect of silver nanoparticles mediated by leech salivary extract on the haematological parameters of wistar albino rats

The effect of leech salivary extract-mediated silver nanoparticles on the haematological parameters of wistar albino rats is revealed in Table 6. All of the treated groups (25, 50 and 100 mg/kgbw) had similar haematological parameters, which were not significantly different (P > 0.05) when compared to the control groups (rats treated with $AgNO_3$ and normal saline).

Table 6: Effect of silver nanoparticles mediated by leech salivary extract on the haematological parameters of wistar albino rats

Treatment (mg/kgbw)	PCV (%)	HB (g/dL)	WBC ($x10^9$)	RBC ($x10^9$)	Platelet ($x10^6$)	MCV (fL)	MCH (pg)	MCHC (g/dL)
25	34.00 ± 4.00[a]	12.70 ± 0.00[a]	5.70 ± 0.60[a]	5.70 ± 0.40[a]	208.00 ± 2.00[a]	66.00 ± 6.00[a]	22.00 ± 2.00[a]	37.80 ± 4.40[a]
50	42.50 ± 2.50[a]	14.15 ± 0.80[a]	4.30 ± 0.20[a]	4.65 ± 0.35[a]	255.00 ± 65.00[a]	92.00 ± 12.00[a]	31.00 ± 4.00[a]	31.80 ± 1.50[a]
100	43.00 ± 5.00[a]	14.50 ± 1.50[a]	4.85 ± 0.80[a]	4.85 ± 0.80[a]	230.50 ± 19.50[a]	91.00 ± 7.00[a]	30.50 ± 2.40[a]	33.30 ± 0.00[a]
Ns	41.00 ± 7.00[a]	13.65 ± 2.30[a]	5.00 ± 1.00[a]	5.45 ± 0.70[a]	215.00 ± 5.00[a]	74.50 ± 2.50[a]	25.00 ± 1.00[a]	33.20 ± 0.10[a]
$AgNO_3$	45.50 ± 3.50[a]	15.15 ± 1.10[a]	4.80 ± 0.40[a]	4.75 ± 0.30[a]	248.50 ± 61.50[a]	96.50 ± 14.50[a]	32.20 ± 4.80[a]	33.30 ± 0.00[a]

Values are expressed as means ± SEM. There is no significant difference (P > 0.05) between values in a column with the same superscript. PCV: packed cell volume, RBC: red blood cells, white blood cells, HB: haemoglobin, MCV: mean cell volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, $AgNO_3$: Silver nitrate, Ns: Normal saline

Effect of silver nanoparticles mediated by leech salivary extract on differential blood count of wistar albino rats

Table 7 shows the effects of AgNPs mediated by leech salivary extract on the differential blood counts in wistar albino rats. All of the treatment groups (25, 50 and 100 mg/kgbw) had similar differential blood counts which do not differ significantly ($P > 0.05$) when compared to the control groups (rats treated with $AgNO_3$ and normal saline).

Table 7: Effect of silver nanoparticles mediated by leech salivary extract on differential blood counts of wistar albino rats.

Treatment (mg/kgbw)	Monocyte	Eosinophil	Basophil	Neutrophil	Lymphocyte
25	4.00 ± 1.00[a]	2.50 ± 0.50[a]	0.00 ± 0.00[a]	52.50 ± 2.50[a]	41.00 ± 4.00[a]
50	3.50 ± 0.50[a]	1.50 ± 0.50[a]	0.00 ± 0.00[a]	46.50 ± 1.50[a]	48.50 ± 1.50[a]
100	5.00 ± 1.00[a]	3.50 ± 0.50[a]	0.50 ± 0.50[a]	50.00 ± 10.00[a]	40.50 ± 10.50[a]
Ns	3.00 ± 0.00[a]	2.00 ± 2.00[a]	0.00 ± 0.00[a]	51.00 ± 2.00[a]	44.00 ± 4.00[a]
$AgNO_3$	4.50 ± 0.50[a]	2.00 ± 1.00[a]	0.00 ± 0.00[a]	34.50 ± 0.50[a]	59.00 ± 1.00[a]

Values are expressed as means ± SD. There is no significant difference ($P > 0.05$) between values with the same superscript. Ns: Normal saline (normal control), $AgNO_3$: Silver nitrate (positive control), SD: standard deviation

Effect of silver nanoparticles mediated by leech salivary extract on liver indices of wistar albino rats

Table 8 shows the effects of silver nanoparticles on the liver indices of wistar albino rats mediated by leech salivary extract. The liver indices levels of all the treated groups were not significantly different ($P > 0.05$) from the control groups.

Table 8: Effect of silver nanoparticles mediated by leech salivary extract on the liver of wistar albino rats.

Treatment (mg/kgbw)	Protein (g/dL)	Albumin (g/mL)	AST (µ/L)	ALT (µ/L)	ALP (µ/L)
25	6.50 ± 0.10[a]	3.40 ± 0.20[a]	37.00 ± 5.00[a]	18.60 ± 2.40[a]	49.00 ± 7.00[a]
50	5.70 ± 0.50[a]	3.70 ± 0.10[a]	41.00 ± 1.00[a]	18.15 ± 2.85[a]	53.00 ± 1.00[a]
100	5.65 ± 1.05[a]	2.40 ± 10.20[a]	42.00 ± 6.00[a]	14.65 ± 2.45[a]	48.50 ± 9.50[a]
Ns	4.90 ± 0.50[a]	2.40 ± 1.40[a]	38.00 ± 6.00[a]	14.10 ± 2.10[a]	44.50 ± 8.50[a]
$AgNO_3$	6.40 ± 0.20[a]	3.45 ± 0.10[a]	47.20 ± 1.00[a]	16.30 ± 0.10[a]	55.00 ± 1.00[a]

Values are expressed as means ± SD. There is no significant difference ($P > 0.05$) between values with the same superscript. Ns: Normal saline (normal control), $AgNO_3$: Silver nitrate (positive control), mg/kgbw: milligram per kilogram body weight, µ/L: micro litre, g/dL: grams per decilitre, mg/dL: milligrams per decilitre, ALP: alkaline phosphatase, ALT: alanine amino transferase, AST: aspartate amino transferase, SD: Standard deviation

Effect of Silver Nanoparticles Mediated by Leech Salivary Extract on Kidney Indices of Wister Albino Rats

The effects of silver nanoparticles mediated by leech salivary extract on the kidney parameters of wistar albino rats are shown in Table 9. Creatinine, urea, potassium, and sodium concentrations in all three treatment groups (25, 50 and 100 mg/kgbw) were not statistically significant.

Table 9: Effect of silver nanoparticles mediated by leech salivary extract on the kidney of wistar albino rats.

Treatment (mg/kgbw)	Urea (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Creatinine (mg/dL)
25	3.90 ± 0.30[a]	145.00 ± 3.00[a]	4.90 ± 0.30[a]	0.50 ± 0.00[a]
50	3.60 ± 0.40[a]	126.00 ± 0.00[a]	4.55 ± 0.35[a]	0.45 ± 0.05[a]
100	3.30 ± 0.50[a]	129.00 ± 17.00[a]	3.75 ± 1.15[a]	0.50 ± 0.30[a]
Ns	3.00 ± 0.40[a]	119.00 ± 9.00[a]	3.30 ± 1.00[a]	0.50 ± 0.10[a]
$AgNO_3$	3.65 ± 0.15[a]	145.00 ± 3.00[a]	4.65 ± 0.05[a]	0.70 ± 0.10[a]

Values are expressed as means ± SD. There is no significant difference ($P > 0.05$) between values with the same superscript. $AgNO_3$: silver nitrate, Ns: normal saline, mg/kgbw: milligram per kilogram body weight, mmol/L: millimoles per litre, mg/dL: milligram per decilitre, SD: Standard deviation

DISCUSSION

In this study, formation of silver nanoparticles using leech salivary extract was viewed by a colour change, from colourless to dark brown. Similarly, Saravana *et al*. (2018) reported that silver nanoparticles exhibited striking colour change from colourless to dark brown in the aqueous solution as a result of the excitation of surface plasmon resonance. The synthesized silver nanoparticles had maximum absorbance peak at 456 nm. Saravana *et al*. (2018) also reported the absorbance of synthesized silver nanoparticles to range from 250 to 600 nm. The particle size of the synthesized silver nanoparticles in this study was revealed to be 98.04 nm. This result corroborates the report of Ganna *et al*. (2020) who evaluated characteristics of *Curcumin* loaded magnesium oxide nanoparticles and described the size of nanoparticles as a particle size ranging from 1 to 100 nm.

The LD_{50} value for a test substance is the dose or concentration required to kill half of a tested population after a certain period of time. The findings from this study revealed that at a concentration limit of 5000 mg/kgbw, the administration of LSE-Ag to rats did not result in mortality or other indicators of toxicity. Although, there were visible signs of fatigue, loss of appetite and decreased activity in the first 24 hours, these signs were reversed on the second day and the animals remained normal thereafter. This, therefore, suggests that the LSE-Ag is relatively harmless acutely. This finding is consistent with Olobayotan and Akin-Osanaiye (2019) findings that biosynthesized silver nanoparticles from *Saccharomyces cerevisiae* had an LD_{50} greater than 5000 mg/kgbw and were therefore non-toxic acutely. Ojo *et al.* (2018) also supported this claim when they observed that the LD_{50} of crude leech salivary extract was above 5000 mg/kgbw.

Body weight changes in rats are a sensitive indicator of the animal's overall health as well as one of the most important symptoms of toxicity. Increases in body weight are seen to be normal, whereas decreases in body weight are thought to be an indication of illness. The animals' body weight increased non-significantly ($P > 0.05$) at 25, 50 and 100 mg/kgbw in this investigation, except at 100 mg/kgbw in week 3, which decreased non-significantly ($P > 0.05$). This could indicate that the LSE-Ag had no effect on the animal's growth, as Luaibi and Qassim (2018) found that silver nanoparticles had no significant ($P > 0.05$) effect on the body weight of rats.

The organ weight is a critical factor in determining whether or not a chemical compound has the ability to cause harm to the organs. It is a highly sensitive indicator of an experimental substance's effect. The absolute weights of kidneys, liver, heart, and lungs do not differ significantly at $P > 0.05$ in this investigation, with the exception of the spleen at 100 mg/kgbw. This suggested that the LSE-Ag had a potential to cause harm to the rats' spleens at doses of 100 mg/kgbw and above. This can be attributed to the rats being hypertensive, which has been reported to exhibit splenomegaly and increased spleen weights may be associated with leukocytosis and extramedullary hematopoiesis (Resendez and Rehagen, 2017). Changes in spleen weight also often reflect xenobiotic-induced alterations in the immune system (Guo and White, 2010). These contrasts the findings of Babayi *et al.* (2018), who reported that the administration of crude leech salivary extract at 25, 50 and 100 mg/kgbw had no significant ($P > 0.05$) effects on the weight of the spleen of rats.

The amount of feed intake in rats gives a good idea of how beneficial a treatment is. In this study, the results of the feed consumption of rats significantly ($P < 0.05$) decreased in week 2 when compared to the control groups (rats treated with $AgNO_3$ and normal saline). Taste is often cited as the factor of greatest significance in food choice, as taste guides food intake. An increase in dietary protein density would decrease intake of carbohydrates and fats with consequent reduction in energy intake (Pezeshki *et al.,* 2016), which may account for the decreased feed consumption observed in week 2 of this study. This finding contrasts Luaibi and Qassim's (2018) reports that the administration of chemically-synthesized silver nanoparticles to rats produced no significant ($P > 0.05$) effect on their feed intake. The findings of the study also contradict those of Ojo *et al.* (2018), who recorded no significant ($P < 0.05$) effect in the feed intake of rats treated with crude leech salivary extract for 28 days.

The fluid intake of rats in the treatment groups significantly ($P > 0.05$) increased in all of the treatment groups when compared to the control groups. The increase could indicate that the LSE-Ag could cause dehydration in rats. This study contradicts the findings of Luaibi and Qassim (2018), who found that the administration of chemically-synthesized silver nanoparticles to rats had no significant ($P > 0.05$) effect on the fluid intake.

The presence or absence of foreign compounds in the blood constituents of a living system is indicated by the haematological parameters such as red blood cells, white blood cells, and platelets, and their investigation aid in identifying several constituents that are of nutritional, physiological and pathological benefits to the status of a living system. Differential blood count indicators: Basophils, Neutrophils, Lymphocytes, Eosinophils, and Monocytes are haematological characteristics that impart immunity to a biological system that protects the body from foreign invaders. The results of this investigation

revealed that there no significant difference (P > 0.05) was recorded for the differential blood counts of the tested animals compared to the control. Thus, the consumption of LSE-Ag could not generate a harmful effect in rats, such as an immunological response or allergic reactions. Babayi *et al.* (2018) made a similar observation that extended administration of crude leech salivary extract produced no significant (P > 0.05) effect on the values of rats' haematological parameters.

For WBC (4 to 10 x 10^9/L), RBC (4.5 to 6.5 x 10^{12}/L) and platelets (150 to 400 x 10^9/L), the values obtained from the rats treated with the LSE-Ag were within the standard reference range as observed in the control group, while PCV (40 to 52 percent), HB (13 to 17 g/dL), and MCHC (30 to 35 g/dL) (Farinde, 2019) were also within the range except at 25 mg/kgbw of the treatment group. Except for the animals treated with 25 mg/kgbw and normal saline, MCH (27 to 32 pg) and MCV (80 to 100 fL) were also within the acceptable range. The values calculated from the parameters of the differential blood count of Wister albino rats treated with the LSE-Ag were in range for Monocytes (2 to 8%) and Eosinophils (1 to 4%), but somewhat below the reference range for Neutrophils (55 to 70%) and Basophils (1 to 4%). Values of lymphocytes (20 to 40 %) were slighty above the reference range (Choladda, 2019).
The liver is an organ found in both humans and animals. It is charged with maintaining homeostasis and regulating the metabolism of harmful chemical substances or medications when they are introduced into the bodily system, which makes it extremely vulnerable to damage. As a result, serum biochemical indices (Alanine amino transferase (ALT), Aspartate amino transferase (AST), Alkaline phosphatase (ALP), Albumin, and Total proteins) are important in predicting the liver's integrity after exposure to medicines, plant extracts, animal metabolites, and other substances including nanoparticles (synthesized from plant and animal metabolites). The changes in the concentration of any of these indices are a common indicator of liver dysfunction.

In the present study, the serum concentrations of ALT, AST, ALP, Albumin, and Total proteins when compared to the control revealed no significant (P > 0.05) differences. This could indicate that the functional integrity of liver of the rats was not impaired during or after treatment. This finding is consistent with Yusuf *et al.* (2018), who found that rats fed *Zingiber officinale* for 28 days showed no significant changes in ALT, AST, ALP, Albumin, or Total proteins levels. The values obtained from the liver indices of wistar albino rats in this investigation were within the conventional reference range for ALT (5 to 30 µ/L), however they were slightly lower. The levels of AST (5 to 30 µ/L), Albumin (35 to 50 g/L), and ALP (50 to 100 µ/L) at 25 mg/kgbw, 50 mg/kgbw, and normal saline treatment groups were slightly lower. When compared to the standard reference at 50 mg/kgbw, 100 mg/kgbw, and normal saline treatment groups, total protein (60 to 80 g/L) was likewise slightly lower (Farinde, 2019).

Animal and human kidneys play a central role in regulating the excretion and re-absorption of chemicals including urea, creatinine, and electrolytes, which can be harmful to the body system if discovered in high concentrations in the serum. The renal function test measures the functionality and dysfunctionality of the kidneys in completing their excretory responsibilities of eliminating harmful chemicals from the body (through filtration). In this study, the treatment of rats with the LSE-Ag resulted in no significant (P > 0.05) changes in serum creatinine, urea, sodium, and potassium concentrations when compared to the control. Thus, this suggests that consuming LSE-Ag at concentrations of 25, 50, or 100 mg/kgbw is unlikely to produce glomerular damage to the kidney. This contradicts the findings of Adeyemi and Adewumi (2014) that recorded significant (P < 0.05) increase in urea, creatinine, and electrolyte levels in rats fed nanoparticles. However, the results obtained in this study supports the findings of Ojo *et al.* (2018), who found that administering of crude leech salivary extract to rats for 28 days had no significant (P > 0.05) effect on electrolytes, creatinine, or urea levels. The creatinine (greater or equal to 4.0 mg/dL) and potassium (less or equal to 2.5 mmol/L or greater than or equal to 6.5 mmol/L) values obtained from the kidney indices of wistar albino rats treated with the LSE-Ag in this study did not fall within the critical standard reference range in humans, but sodium (135 to 145 mmol/L) values were within the range, while urea level (1.2 to 3.0 mmol/L) was slightly above the standard range except for the group treated with normal saline.

CONCLUSION

The LSE-Ag produced in this study had a wavelength of 456 nm and a size of 98.04 nm. The LSE-Ag had an oral LD50 of above 5000 mg/kgbw. No changes in haematological parameters (PCV, MCH, HB, MCV, MCHC, and Platelets), white blood cell and differential blood count (Eosinophils, Monocytes, Basophils, Neutrophils, and Lymphocytes) were seen after administering LSE-Ag orally for 21 days. As the LSE-Ag treatment progressed, the rats' feed intake decreased while their fluid consumption increased. At 100 mg/kgbw, the LSE-Ag showed no effect on the absolute weight of the organs (lungs, liver, heart, or kidneys) of rats, and significantly (P < 0.05) increased the weight of the spleen. The rats' serum total protein, aspartate amino transferase, alanine amino transferase, alkaline phosphate, sodium, potassium, creatinine, and urea levels produced no toxic effects by the chronic administration of

the LSE-Ag. Therefore, the LSE-Ag is relatively safe and could be a useful drug candidate. Further studies should be undertaken to include the effects of LSE-Ag on organs of wistar rats.

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