ANTIBACTERIAL ACTIVITIES OF LEECH SALIVARY EXTRACT-MEDIATED SILVER NANOPARTICLES AGAINST SOME PATHOGENIC BACTERIA

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Abstract

Leech salivary extract (LSE) and its salivary extract-mediated silver nanoparticles (LSE-Ag) were screened for its antibacterial activities against Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Klebsiella species using agar well diffusion method. The activity was compared with augmentin. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of LSE-Ag were assayed using micro broth dilution method with tetrazolium dye as indicator. The LSE-Ag was biologically synthesized and characterized by ultraviolet spectroscopy and a nanosizer. The wavelength of the LSE-Ag was set at 456 nm while the size was at 98.04 nm. The crude LSE was inactive on all the test isolates while the LSE-Ag inhibited the growth of P. aeruginosa with zones of inhibition of 8.3 ± 0.88 mm and 12.3 ± 0.88 mm at 100 µL and 200 µL respectively and Klebsiella sp with the zones of inhibition of 12.0 ± 0.57 mm and 12.3 ± 0.33 mm at 100 µL and 200 µL respectively. The MIC of LSE-Ag was at 100 µL for P. aeruginosa and Klebsiella sp., while the MBC for both organisms were at 200 µL. Staphylococcus aureus and E. coli were resistant to the LSE-Ag. The results obtained in this study suggest that LSE-Ag could be used in treating infectious diseases caused by Pseudomonas aeruginosa and Klebsiella species.

Keywords*:* Antibacterial activity; Leech; Leech salivary extract-mediated silver nanoparticles (LSE-Ag); Minimum bactericidal concentration (MBC), Minimum inhibitory concentration (MIC)

Introduction

Pathogenic microorganisms especially bacteria that cause diseases have always been a major threat to human lives and hospitalized patients (Sarmah *et al*., 2018). It is one of the cogent challenges faced by medical professionals. Early and accurate diagnosis and treatment of infectious diseases can be challenging, due to the toxic nature of some chemically synthesized drugs and resistance shown by pathogenic strains against conventional antibiotics Pathogenic organisms use different techniques to evade conventional drugs and these techniques may include: biofilms and/or capsule formation, the use of proton efflux pump to mention but a few. This in turn adds up to the virulence of an organism (Pigłowski, 2019).

In spite of the progress accomplished in treatment of infectious diseases in recent decades, the rates of morbidity and mortality have been on the rise. The Nigeria centre for disease and control (NCDC) in 2019 stated that infectious diseases are the leading cause of hospitalisation and death in Nigeria. The rate of infectious diseases in Nigeria and Africa at large is on the increase. The rate of antibiotic resistance, toxic nature of some chemically synthesized drugs as well as high cost of drugs in relation to poverty in the continent of

Africa have contributed immensely to the ineffectiveness in the treatment of infectious diseases (Chattu & Yaya, 2020). Attention is now being turned to nature, in sourcing for safe, cheap and reliable drugs in combating infectious diseases.

Leeches belongs to the phylum *Annelida.* They are worms that suck blood and are hermaphroditic in nature. More than 600 species have been identified and fifteen of those have been classified as medicinal (Wollina *et al*., 2016). They are locally called *'Mosasaku'* in Hausa, *'Mujemuje'* in Yoruba, '*Tuturu'* in Nupe and '*Idu'* in Igbo. Medicinal leeches belong to the order 'Arhynchobdellida' and family 'Hirudinidae'. Examples are *Hirudo medicinalis*, *H. verbena* and *H. orientalis* (Wollina *et al*., 2016). Other species of Leeches have been studied and a number of proteins and peptides have been found in their salivary extract. Leeches have been used for treatment of a wide range of diseases such as dental infections, skin diseases, wound healing, nervous system abnormalities, problem of urinary and reproductive system, and inflammation (Ghawi *et al*., 2012).

Nanoparticles (NPs) are particles that range in size from 1 to 100 nanometers (nm). They are synthesized mostly from transition metals in the periodic table. A wide variety of approaches (such as chemical, physical and biological or biogenic) have been used for the synthesis of nanoparticles (Patra & Baek, 2014). However, biogenic reduction of metal precursors in producing its corresponding NPs has proven to be eco-friendly, less expensive and free of chemical contaminants. Natural products such as plant extract or animal secretions that are proteineous, and embedded with natural stabilizing, growth terminating and capping compounds have been a good source of biogenic reduction of metallic particles to nanoparticles. Metallic nanoparticles produced by biogenic reduction such as silver nanoparticles (AgNPs) have been revealed to have antimicrobial properties against some pathogenic organisms. Its application also extends into biomedical sciences for bio-imaging, drug transport, cancer treatment, medical diagnosis and sensor construction due to their unique properties, structure and size (Nadaroğlu *et al*., 2017). The combination of leech saliva and silver nitrate lead to formation of silver nanoparticles with minute size that enhance it to act as a drug carrier incorporating bioactive substances into its matrix which may inhibit the growth of pathogens. The aim of the study therefore, is to evaluate the antibacterial activity of crude leech salivary extract and leech salivary extract-mediated silver nanoparticles against some pathogens.

Materials and Methods

Collection and Identification of Leeches: Leeches were collected with the aid of a scooping net at the shorelines of fresh water Dam, in Bosso, Niger State and Panda Development Area, Nassarawa State, Nigeria in the month of June, 2019. The worms were identified by a Zoologist, Dr. Azubuike in the Department of Animal Biology, Federal University of Technology, Minna, Niger State as *Hirudo medicinalis*.

Laboratory Maintenance of Leeches: The leeches were maintained in well-aerated plastic containers filled with non-chlorinated water (borehole water), at room temperature (25 ± 2 °C) throughout the period of study. The water was frequently changed at 2 days interval and feeding of the leeches with cow blood was carried out 3 weeks interval after the extraction of their saliva (Abdualkader *et al*., 2014).

Extraction of Leech Saliva: The extraction of leech saliva was carried out by Ice-shocking method as described by Abdualkader *et al*. (2014) and Ojo *et al*. (2018). Two to three pieces of leeches were placed in a test tube and the test tube was positioned in a bowl packed with ice blocks for 20 minutes. Leeches were completely paralyzed and forced to regurgitate their intestinal content into a sterile test tube. The saliva was aspirated using a

sterile hypodermic needle and syringe, and then transferred into sterile screw capped containers and preserved at −4 °C in a refrigerator.

Source and Identification of Test Organisms: The test organisms used in this study were isolated from blood from patients admitted at General Hospital Minna, Niger State. The clinical isolates were identified using the method described by Cheesbrough (2010). Morphological and biochemical tests (such as Gram's staining, catalase, oxidase, coagulase, citrate, urease, starch hydrolysis, methyl red, indole, and sugar fermentation) were used in the isolates' identification. The organisms were maintained in slants until required.

Antibacterial Assay

Standardization of test organisms: Following the method of National Institute for Pharmaceutical Research and Development, NIPRD (2006), 0.2 mL of an overnight culture of each test organism was dispensed into 20 mL of freshly prepared sterile nutrient broth and incubated for 5 hours to standardize the culture to 10^6 cfu/mL. The absorbance of the standardized culture was obtained using a spectrophotometer at 625 nm.

Screening for antibacterial activity of crude leech salivary extract: The crude leech salivary extract at one hundred micro litres (100 µL) and two hundred micro litres (200 µL) respectively, were assayed for antibacterial activity on the test isolates using agar well diffusion technique described by Clinical and Laboratory Standard Institute, CLSI (2016). Muller Hinton agar (MHA) with a thickness of 4mm was prepared. Standardised isolates (a loopful) were seeded on the agar plate with the aid of a sterile wire loop. Four holes were bored on the plate using a sterile cork borer (5 mm in diameter) and the bases of the holes were sealed with sterile molten agar. Two concentrations of 100 µL and 200 µL of crude Leech salivary extract were dispensed into the first two holes (labeled 'A and B') while the other two holes were filled with a standard broad spectrum antibiotic (Augmentin (8 µg/mL) labeled '+') and sterile distilled water (labeled '-'). Afterwards, the plates were made in triplicates and incubated at 37 °C for 24 hours and zones of inhibition were observed, measured and recorded at the end of the incubation period.

Synthesis of leech salivary extract-silver nanoparticles conjugate: The method of Jaganathan *et al*. (2016) were used for the synthesis of silver nanoparticles from LSE. A solution of silver nitrate (2 mM) was prepared by dissolving 0.034 g of the salt in 100 mL of sterile distilled water. A measured quantity of 10 mL of the prepared 2 mM silver nitrate solution was dispensed into a conical flask and 1 mL of the leech salivary extract (LSE) was added to it, to make up a ratio of 1:10. The resulting mixture was placed under the sun for 30 minutes. A dark brown colouration was observed in the reaction mixture.

Screening for antibacterial activities of leech salivary extract- mediated silver nanoparticles: The LSE-Ag at 100 µL and 200 µL were assayed for antibacterial activities on the test isolates using agar well diffusion technique employed by Clinical and Laboratory Standard Institute, CLSI (2016) and described above for antibacterial activity of crude leech salivary extract.

Determination of minimum inhibitory concentration of leech salivary extract-mediated silver nanoparticles: The minimum inhibitory concentration (MIC) of the leech salivary extract-mediated silver nanoparticles was determined using the micro broth dilution method desribed by Ojo *et al*. (2018). Two hundred micro litres (200 µL) of sterile Muller Hinton's broth was dispensed into a micro titre plate (from well 2 to 12) with the aid of a multiple channel pipette. Two hundred micro litres of LSE-Ag was then dispensed into well 1 and well 2. Two hundred micro litres of the resulting mixture of broth and LSE-Ag in well 2

was pipetted and dispensed into well 3 (making a double fold dilution). This was carried out for well 4 through well 10. Two hundred micro litres of standardized culture of *Pseudomonas aeruginosa* was dispensed into well 1 through well 9 and well 11 respectively. The plate was incubated at 37 °C for 24 hours. At the end of the incubation period 50 µL of tetrazolium dye was added to the wells and further incubated for 20 minutes to check for colour change from yellow to purple. The same procedure was repeated for the MIC of the nanoparticles for *Klebsiella* spp (Ojo *et al*., 2018). Well 1, 10, 11 and 12 served as the nanoparticles sterility control, organism viability control and broth sterility control respectively.

Determination of minimum bactericidal concentration of leech salivary extract-mediated silver nanoparticles: The MBC of the LSE-Ag was determined using the method described by Omeje and Kelechi (2019). A loopful of content from MIC well was dispensed into fresh Mueller Hinton agar and incubated at 37 °C for 24 hours. Absence of growth was recorded as bactericidal while presence of growth was taken as bacteriostatic.

Data Analysis: Data generated in this study were expressed as the mean value ± standard deviation. Comparisons between different groups were carried out by analysis of Variance (ANOVA). Significant differences between the control and experimental groups were determined at 95% level of significance by Duncan Multiple Range Test (DMRT) using the Statistical Package for Social Sciences (SPSS) version 21.

Results

Morphological Characteristics and Identities of Test Isolates:

Table 1 shows the characteristics and identities of the test isolates. The organisms isolated included: *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella* spp.

Antibacterial Activities of Crude Leech Salivary Extract on Test Isolates:

Table 2 revealed the antibacterial activity of the crude leech salivary extract (undiluted) on the test isolates. The crude leech salivary extract was unable to inhibit the growth of all the test isolates at 100 µL and 200 µL. The standard antibiotics (Augmentin) used as the positive control inhibited the growth of the test isolates at 8 µg/mL with zones of inhibition of 20.00 ± 0.33 mm, 25.00 ± 0.57 mm, 27.00 ± 0.88 mm and 31.00 ± 1.20 mm against *Staphylococcus aureus*, *Klebsiella* spp, *Escherichia coli* and *Pseudomonas aeruginosa* . Distilled water which served as the negative control showed no inhibition on the growth of the test isolates.

Table 1: Morphological Characteristics and Identities of Test Isolates

GR	SHAPE	MO	CA	CO	CI	SH	IN	MR	H_2S	OX	UR	Sugar Fermentation					Suspected Organisms
												G	F	L	M	S	
G-	Rod	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	*P. aeruginosa*
G-	Rod	-	+	-	+	+	-	-	-	-	+	+	-	+	+	+	*Klebsiella* spp
G-	Rod	+	+	-	-	+	+	+	-	-	+	+	-	+	-	+	*E. coli*
G+	Cocci	-	+	+	+	+	-	+	+	-	+	+	+	+	+	+	*S. aureus*

GR: Gram's reaction, G+: Gram positive, G-: Gram negative, MO: Motility test, CO: Coagulase, CA: Catalase, CI: Citrate utilization, SH: Starch hydrolysis, IN: Indole, MR: Methyl red, H_2S: Hydrogen sulphide production, OX: oxidase, UR: Urease, G: glucose, F: Fructose, L: Lactose, M: Maltose, S: Sucrose.

Table 2: Antibacterial Activities of Crude Leech Salivary Extract on Test Isolates

Test isolates	Diameter Zones of inhibition (mm)			
	LSE (100 µL)	LSE (200 µL)	Dw (100 µL)	Aug (8 µg/mL)
P. aeruginosa	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	31.00 ± 1.20[b]
Klebsiella spp	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	25.00 ± 0.57[b]
E. coli	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	27.00 ± 0.88[b]
S. aureus	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	20.00 ± 0.33[b]

Values are means ± standard deviation. Values with the same superscript in a row have no significant difference at (P > 0.05). LSE: Leech salivary extract, µL: Micro litre, Dw: Distilled water, Aug: Augmentin, µg/mL: Microgram per millilitre, mm: millimetre

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 (Figure 1a). The size of the particle was 98 nm (Figure 1b).

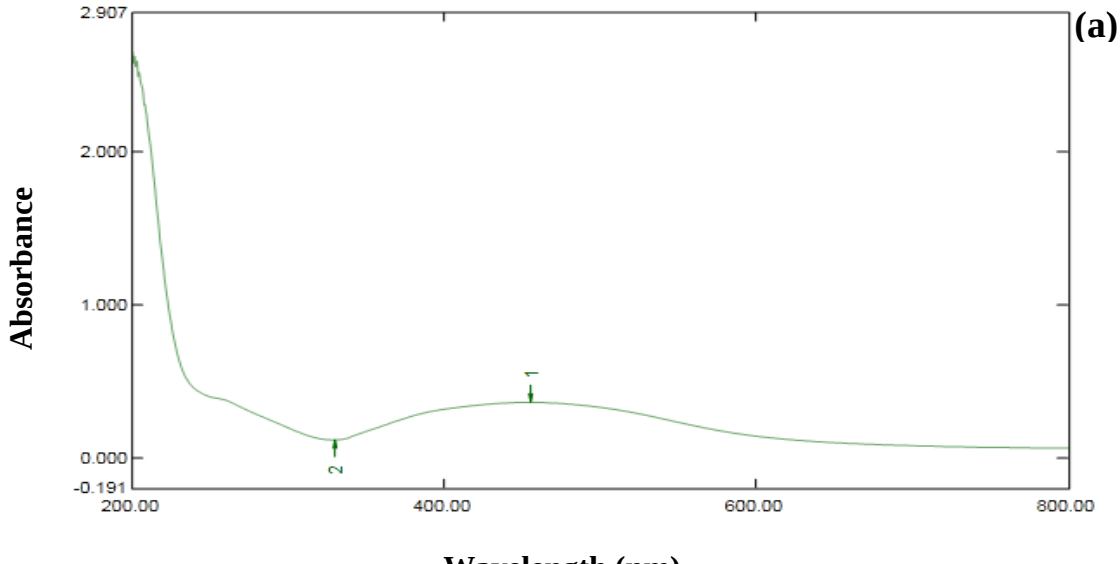

Figure 1a: Ultraviolet-Visible-Spectrum of Leech Salivary Extract-Mediated Silver Nanoparticles

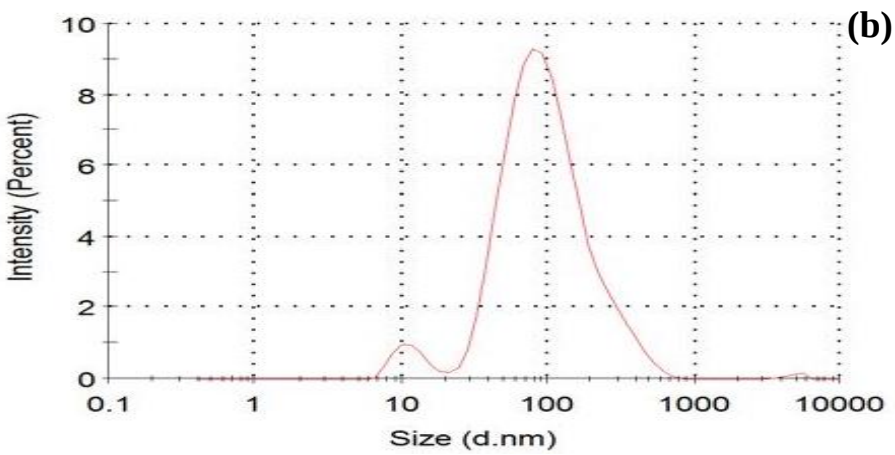

Figure 1b: Zeta Nano Size Distribution Pattern of Leech Salivary Extract-Mediated Silver Nanoparticles

Antibacterial Activities of Leech Salivary Extract-Mediated Silver Nanoparticles on Test Isolates:

The antibacterial activities of Leech salivary extract-mediated silver nanoparticles against the test isolates at 100 µL and 200 µL respectively are shown in Table 3. The diameter zones of inhibition produced by LSE-Ag are 8.33 ± 0.88 mm and 12.33 ± 0.33 for *Pseudomonas aeruginosa* at 100 µL and 200 µL respectively. It produced 12 ± 0.57 mm and 12.33 ± 0.33 mm for *Klebsiella* sp at 100 µL and 200 µL respectively while at the same concentration *Escherichia coli* and *Staphylococcus aureus* were resistant to it. The activity of the conjugate was compared with augmentin which produced zones of inhibition of 20.33 ± 0.33 mm, 25.00 ± 0.57 mm, 27.33 ± 0.88 mm and 31.33 ± 1.20 mm, for *Staphylococcus aureus*, *Klebsiella* sp, *Escherichia coli* and *Pseudomonas aeruginosa*.

Table 3: Antibacterial Activities of Leech Salivary Extract-Mediated Silver Nanoparticles on Test Isolates

Test isolates	Zones of inhibition (mm)			
	LSE (100 µL)	LSE (200 µL)	Dw (100 µL)	Aug (8 µg/mL)
Pseudomonas aeruginosa	8.33 ± 0.88^{b}	12.33 ± 0.88^{c}	0.00 ± 0.00^{a}	31.33 ± 1.20^{d}
Klebsiella species	12.33 ± 0.33^{b}	12.00 ± 0.57^{b}	0.00 ± 0.00^{a}	25.00 ± 0.57^{c}
Staphylococuss aureus	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	20.33 ± 0.33^{b}
Escherichia coli	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	27.33 ± 0.88^{b}

Values are means ± standard deviation. Values with the same superscript in a row have no significant difference at (P > 0.05). LSE: Leech salivary extract, µL: Micro litre, Dw: Distilled water, Aug: Augmentin, µg/mL: Microgram per millilitre mm: millimetre.

Minimum Inhibitory and Minimum Bactericidal Concentrations of Leech Salivary Extract-Mediated Silver Nanoparticles on Test Isolates:

Table 4 revealed the minimum inhibitory and bactericidal concentration of leech salivary extract-mediated silver nanoparticles on test isolates. The MIC of the LSE-Ag against *Pseudomonas aeruginosa* and *Klebsiella* spp was at 100 µL while the MBC was 200 µL respectively.

Table 4: Minimum Inhibitory and Bactericidal Concentration of Leech Salivary Extract Silver Nanoparticles on Test Isolates

Test Isolates	Concentrations of LSE-AgNp (µL)									
	200	100	50	25	12.5	6.25	3.12	1.56	MIC	MBC
P. aeruginosa	+	+	-	-	-	-	-	-	100	200
Klebsiella spp.	+	+	-	-	-	-	-	-	100	200

+: Activity, -: No activity, LSE-AgNP: leech salivary extract silver nanoparticles, MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration.

Discussion

In the present study, the test isolates were catalase positive (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella* spp), oxidase negative (*E. coli*, *S. aureus* and *Klebsiella* spp), H_2S negative (*P. aeruginosa*, *Klebsiella* sp and *Escherichia coli*), urease hydrolyser (*E. coli, S. aureus* and *Klebsiella* spp), glucose, lactose and sucrose fermenters (*E. coli, S. aureus* and *Klebsiella* spp), starch hydrolyser (*E. coli*, *S. aureus* and *Klebsiella* spp). These observations are similar with the findings of Alhubail *et al*. (2020) that identified similar organisms using biochemical tests. Gram staining of the test isolates revealed Gram negative rods (*P. aeruginosa, E. coli* and *Klebsiella* sp) and Gram positive coccus (*S. aureus*). This is similar to the report of Abdallah *et al*. (2016) who used Gram staining to classify the same organisms.

The crude leech salivary extract (LSE) showed no inhibitory effect on the test isolates. This could be as a result of low concentrations of bioactive molecules in the extract (Ashraf & Bakri, 2018). This result contradicts the report of Malik *et al*. (2019) who reported that, leech salivary extract showed inhibitory effect on some of the test isolates used in this study. 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 which is due to excitation of surface Plasmon resonance. The maximum absorbance peak with UV-vis sprectrum was 456 nm. Similarly, Saravana *et al*. (2018) also reported absorption spectra of silver nanoparticles found in the reaction media to range from 250 to 600 nm. The size of the particle synthesized in this study, was revealed to be 98.04 nm. This result is in agreement with the report of Ganna *et al*. (2020) who described the size of nanoparticles as a particle size ranging from 1 to 100 nm when evaluating the characteristics of *Curcumin* loaded magnesium oxide nanoparticles.

The Leech salivary extract-mediated silver nanoparticles (LSE-Ag) were observed to inhibit the growth of *P. aeruginosa* and *Klebsiella* spp while *S. aureus* and *E. coli* were resistant to it. Ojo *et al*. (2018) reported that bioactive compounds such as proteins and antimicrobial peptides (AMP), 4-bromobutyric acid, 6, 17-Octadiene-1-ol acetate and octahydro-1, 4, 9, 9-tetramethyl are responsible for LSE's activity. The observed activity in the LSE-Ag as against the crude LSE counterpart could be due to the submicron (minute) size of silver nanoparticles which enables it to act as a drug carrier by incorporating bioactive substances (hydrophilic and hydrophobic) into its matrix. The bioactive substances in the matrix of the LSE-Ag may inhibit the growth of microorganisms by reacting with thiol group of some enzymes or respiratory enzymes leading to death or the release of reactive oxygen species which attacks microbial cells or accumulates

itself in pits that form on the cell wall and punching holes in the membrane of microorganisms leading to denaturation and cell death (Malik *et al.,* 2019; Shikha *et al*., 2020).

Conclusion

The present investigation revealed the potential of silver nanoparticles obtained from leech saliva as a rich source of antibacterial agent.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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