THE ANTIBACTERIAL ACTIVITIES OF *EUDRILUS EUGENIAE*-MEDIATED BIOSYNTHESIZED SILVER NANOPARTICLES AGAINST ISOLATES FROM PATIENTS WITH DIABETIC FOOT INFECTION

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Abstract

Individuals with diabetic foot infection are often infected with multidrug resistant organisms due to chronic course of wound, neuropathy and peripheral vascular disease. The antibacterial potentials of crude extract and extract mediated silver nanoparticles of *Eudrilus eugeniae* were investigated against isolates from patients with diabetic foot infection using agar well diffusion method. Saponins, proteins, terpenoids, phenols and steroids were detected in the crude extract and fractions. Crude ethanol, ethyl-acetate and aqueous fractions at 100 mg/ml and 200 mg/ml showed very weak inhibitory effect against *Psuedomonas aeruginosa*, *Staphylococcus. aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Escherichia coli*. Dark brown aqueous mediated silver nanoparticles (AgNPs) were formed which gave maximum absorbance at 408 nm. The synthesized particles were spherical, less aggregated and contained alkanes, esters and alcohol functional groups. The zones of inhibition produced by the AgNPs (at 1:4 dilution) were 15.33±0.58 mm and 17.67±0.58 mm against *Escherichia coli* and *Pseudomonas aeruginosa* while at 1:5 dilution, 12.33±2.08 mm, 14.67±0.58 mm and 15.33±1.16 mm were zones of inhibition produced against *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* respectively. The MIC of the AgNPs against *P. aeruginosa* was at 6.12 mg/mL while it was at 12.5 mg/mL against *E. coli* and *K. pneumoniae.* The extract was bacteriostatic on the test isolates. The results of the findings showed that the extract mediated silver nanoparticles have enhanced activity against the isolates compared to the crude extract. Data generated suggest that AgNPs synthesized from *E. eugeniae* could be a rich source of antibacterial agent.

Keywords: Antimicrobial activity; crude extract and fractions; diabetic foot infection; *Eudrilus eugeniae*; silver nanoparticles.

Introduction

The term diabetes describes a group of metabolic disorders characterized and identified by the presence of hyperglycaemia (World Health Organization, WHO, 2019). Diabetes mellitus is a chronic metabolic disorder imparting loss in health and economic burden on the patients (Kavya & Alain, 2020). According to International Diabetes Federation, in 2017 about 451 million people were diabetic and figure is expected to increase to 693 million people in 2045 (Cho & Malanda, 2018; Pouya *et al*., 2020). The risk for developing foot ulcer is 25 % high in patients with diabetes and it was reported that in every 30 seconds one lower limb amputation in diabetic patients occurs around the world. A meta-analysis study published recently showed that the world wide prevalence rate of diabetic foot infection is 6.3 % (Pouya *et al.,* 2020).

Diabetic foot infection (DFI) represents a severe complication of long-standing diabetes mellitus. It is an open wound that occurs in approximately 15 percent of patients with diabetes, commonly located at the lower limb precisely, the foot. Diabetic ulcer develops due to poor blood circulation following diabetes, although, other areas of the body are prone to such ulcer. Inadequate treatment of diabetic foot infection could lead to the spread of the infection from foot ulcer to the bone, thus causing osteomyelitis that exposes the patient to the risk of amputation, bacteremia and death (Benjamin *et al*., 2019; Lauri *et al*., 2020).

Patients with diabetic foot infection are often infected with multidrug resistant organisms due to chronic course of the wound, inappropriate antibiotic treatment, frequent hospital admission, neuropathy, nephropathy and peripheral vascular disease (Priya *et al*., 2019; Vanessa *et al.,* 2020). For example methicillin-resistant *Staphylococcus aureus* infections delay wound healing time, increase hospitalization stay, increase the need for surgical procedures and result in treatment failure (Vanessa *et al.,* 2020). Some of the microorganisms responsible for diabetic foot infection include *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus* spp. *Escherichia coli, Enterobacteriaceae, Acinetobacter* species *and Klebsiella pneumonia* (Priya *et al*., 2019; Vanessa *et al.,* 2020). Treatment of diabetic foot infection results to devastating economic crisis for the patient's families and society at large (Alok *et al.,* 2018; Bus *et al*., 2020). Furthermore, deep soft-tissue infections in diabetic persons can be associated with gas- producing Gram negative Bacilli (Lauri *et al.,* 2020).

Some factors contributing to development of the diabetic foot infection include walking barefooted, delay in reporting to the medical center for clinical assessment, impaired sweating, dry and cracked skin, toenail infections and foot abnormalities such as Charcot foot. Diabetes cause impairment in the body production of insulin (a hormone that enable the cells to obtain and utilize glucose from the blood stream for energy generation) and this disruption to insulin production makes it more difficult for the body to manage blood glucose levels. When blood glucose remains permanently high, it impairs the function of white blood cells (WBCs). The white blood cells are central to the role of immune system. When WBCs are unable to function properly, the body is less able to fight bacteria and heal wounds. Also uncontrolled diabetes may develop poor circulation. As circulation slows down, blood moves slowly this makes it more difficult for the body to deliver nutrients to wound site. As a result, the injuries heal slowly or may not heal (Patrick, 2019). Prolonged treatment of infectious diseases such as diabetic foot infection with synthetic antimicrobial agents had lead to resistance of microorganisms to the antibiotics. It becomes vital to source for a well-tolerated more effective and affordable antimicrobial agents.

Eudrilus eugeniae is a tube like, segmented worm found in the phylum Annelida. It belongs to the family Eudrilidae, genus *Eudrilus* and species *eugeniae* (Sethulakshmi *et al.,* 2018). *Eudrilus eugeniae* is called 'Earthworm' in English, *'Ogbummiri'* in Igbo, '*Tana*' in Hausa and *'Ekolo'* in Yoruba, '*Egwongi*' in Nupe and '*Mbuombulo*' in Gbagi. Earthworms are commonly found in soil and play a major role in the proper functioning of the soil ecosystem. The medical value of earthworms had been known for centuries. The extracts prepared from earthworm tissues have been used for the treatment of numerous diseases since they are valuable source of proteins, peptides, enzymes and physiologically active substances. Earthworm has been recognized in oriental medicine as anti-inflammatory, analgesic and antipyretic agent (Patil & Biradar, 2017; Wang *et al.,* 2018; Augustine *et al.,* 2018). Earthworm

surface excreta were found to have potent antimicrobial activity (Hussain *et al.*, 2021). It also has anti-coagulatory or fibrinolytic activity which results in the facilitation of blood circulation. Earthworms were used as a traditional Chinese medicine and have been applied pharmacologically and clinically. It has healing effects on wounds. It decreases the wound healing time and reduced ill- effects of inflammation as determined by macroscopy, histopathology, hematology and immunohistochemistry parameters (Zhen-hen *et al.,* 2018). To further enhance the antimicrobial activities of the earthworm extract, silver nanoparticle was synthesized from it. Silver nanoparticles is a carrier, owing to its high targeting potentials and efficient delivery, it moves the antimicrobial agent to the targeted site of the cells of the microorganisms (Chengzhu *et al*., 2019).

Silver nanoparticles are nanoparticles of silver between 1 nm and 100 nm in size. While frequently described as being silver, some are composed of a large percentage of silver oxide due to their large ratio of surface to bulk silver atoms. Numerous shapes of nanoparticles can be constructed depending on the application at hand. Commonly used silver nanoparticles are spherical in shapes but diamond, octagonal and thin sheet are also popular. The green synthesis of silver nanoparticles (AgNPs) is considered an eco-friendly technology leading to a reduction in the generation of hazardous substances. Silver ions and silver based compounds are highly toxic to microorganisms, including important species of pathogenic bacteria (Johnston *et al.*, 2016). Silver nanoparticles have emerged with diverse medical applications including silver-based dressings and silver-coated medicinal devices, such as nano-gels and nano-lotions. All bacteria use an enzyme as a form of chemical lung in order to metabolize oxygen. Silver ions cripple the enzyme and stop the uptake of oxygen. This effectively inhibits the growth of the bacteria, killing it within 6 minutes and leaving the surrounding tissue or material unaffected (Johnston *et al.*, 2016). These potentials of silver nanoparticles necessitated the synthesis using earthworm extracts as both function in synergy in inhibiting the growth of microorganisms. Therefore, the aim of this study was to investigate the antibacterial activities of silver nanoparticles synthesized from *Eudrilus eugeniae* on isolates from patients with diabetic foot infection.

Materials and Methods

Collection and identification of earthworm

Matured earthworms were collected in Bosso, Bosso Local Government, Minna, Niger State, Nigeria, in the month of July, 2019. The earthworms were kept in a dark container which was half filled with soil to create a familiar environment and covered with a perforated lid. The worms were identified by a zoologist, Dr. K. A. Adeniyi, of the Department of Animal Biology, Federal University of Technology, Minna, as *Eudrilus eugeniae.*

Preparation and extraction of earthworm

The earthworms were washed under running tap water to remove soil and mucus from the surface of the body. They were rewashed with sterile distilled water to further remove all the dirt. The worms (300 g) were freeze dried in FGJ-18 Freeze dryer at -28 °C. The dried worms were pulverized to obtain the powdered form using ES-242 eurosonic electric blender. It was stored in a sterile container until required for use. The pulverized worm powder was successively and exhaustively extracted using 70 % ethanol (1.5 L) for 72 h, ethylacetate (1 L), distilled water (1 L) and n- hexane (1 L) by cold maceration method. The mixture of ethanol and powdered earthworm was filtered using Whatman filter paper number 1 and half of the filtrate was evaporated to dryness using rotary evaporator at 50 °C and freeze dryer at -28 °C to obtain crude ethanol extract (E), 32g.

The remaining filtrate was dispensed into a separating funnel and One liter of ethyl acetate was intermittently added and left f or 24 h. The soluble ethyl acetate extract was released into a beaker by adjusting the regulating knob of the separating funnel, evaporated to dryness and labeled Ec. The crude ethanol extract was re-extracted in 1 L of n-hexane for another 24 hours and soluble n-hexane portion (NH) was removed and evaporated to dryness. The crude ethanol extract was extracted with 1 L of ethylacetate and finally 1 L of sterile distilled water for 24 h to obtain the ethylacetate and aqueous portion (A), (Samatra *et al.,* 2017). Prior to assay, all extracts were dried at 40 °C in an oven to remove the residual effects of the extracting solvent which may interfere with the results.

Bioactive screening of *Eudrilus eugeniae* extracts
Quantitative and Qualitative bioactive screening was carried out on all the crude extracts and fractions of *E. eugeniae* to detect the presence or absence of various secondary metabolites using standard methods (Roghini & Vijayalakshmi, 2018).

Collection and identification of test organisms
Ethical approval was obtained from research and ethics committee of General Hospital and IBB Specialist Hospital, Minna, Niger State. Test organisms were clinical strains obtained from foot ulcer of diabetic patients attending General Hospital and IBB Specialist Hospital, Minna. The disninfected ulcer area were swabbed with sterile swab sticks and the swabs were stored in sterile normal saline containers and were transported to Vaccine laboratory, Center for Genetic Engineer and Biotechnology, Federal University of Technology, Minna within one hour of collection. The swabs were immediately inoculated on nutrient agar and incubated at 37℃ for 24 h. Subsequently, the isolates were subcultured on blood agar (BA) and MacConkey agar (MCA). The resulting colonies were Gram stained and further characterized using standard biochemical tests such as catalase, coagulase, indole, hydrogen sulphide production, haemolysis production, sugar fermentation, methyl red test, starch hydrolysis, citrate test and Voges-Proskauer. The organisms were maintained on nutrient agar slants at 4 ℃ until required for use (Cheesebrough, 2010).

Standardization of bacteria
The method of National Institute for Pharmaceutical Research and Development (NIPRD), (2018) was employed in standardizing the five bacterial isolates. Five milliliter of sterile nutrient broth was dispensed into test tubes which were inoculated with the isolates. They were incubated for 24 h at 37 ℃. A measured quantity of 0.2 mL of the overnight culture of each bacterium was dispensed into 20 mL of sterile nutrient broth and incubated for 5 h at 37 ℃ to standardize the culture to 10^6 cfu/mL.

Antibacterial screening of crude extract and fractions
The antimicrobial activity of the extract and fractions of *Eudrilus eugeniea* at 200 mg/mL were assayed on the standardized organisms using agar diffusion method as described by Clinical and Laboratory Standard Institute (2016). Muller Hinton agar was dispensed in petri dishes and allowed to solidify. Extract (0.2 g) was reconstituted 0.2 g in 5 mL of sterile distilled water for the polar extract (aqueous) while for the non-polar and mid polar extracts (hexane and ethyl acetate), the extracts were first homogenized with 1 mL of tween 80 and then added to 4 mL of sterile distilled water. The extracts were stirred and agitated vigorously. The standardized test bacteria were seeded on the surface of Muller Hinton agar plates using sterile inoculating loop. By means of sterile cork borer, 4wells were bored on each plate aseptically (6 mm in diameter). The base of each well was sealed with sterile molten agar. The reconstituted extracts (0.2 mL each) were dispensed into the wells aseptically using sterile syringe and needle. The plates were prepared in triplicates. Amoxicillin and Clavulanate Potassium (0.05 mg/mL) was used *as* the positive control and the solvents were used as negative control on different plates. The plates were incubated at 37 ℃ for 24 h.
The antibacterial activity was determined by measuring the diameter of zone of inhibition around the wells in millimeter. Control plates which include organism viability control (OVC), medium sterility control (MSC), extract sterility control (ESC) were made in parallel and observed.

Biosynthesis of silver nanoparticle using *Eudrilus eugeniae* extract and fractions
Aqueous solution of silver nitrate (2 mM) was prepared by dissolving 0.0358 g of silver nitrate ($AgNO_3$) in 100 mL of sterile distilled water. One gram of ethanol extract was dissolved in 100 mL of distilled water. Dilution and pH were optimized in the study. Forty milliliters of the $AgNO_3$ solution was added to 10 mL of ethanol extract solution of *Eudrilus eugeniae*. Fifty milliliters of silver nitrate solution was added to 10 mL of ethanol extract solution to obtain 4:1 dilution, and were stirred thoroughly using magnetic stirrer at room temperature. The pH of the solution was adjusted to 12 using NaOH solution and the mixture was exposed to direct sunlight for 15 minutes. The same procedure was carried out for the synthesis of silver nanoparticles using ethyl acetate, n-hexane and aqueous fractions respectively (Jaganathan *et al.,* 2016).

Characterization of the synthesized silver nanoparticles
The synthesized silver nanoparticles were characterized using UV-visible spectrophotometry, scanning electron microscopy, X-ray diffraction analysis, fourier transformed infrared and energy dispersive x-rayspectroscopy as described by Saher *et al.* (2019).

Antibacterial screening of synthesized silver nanoparticles
The antimicrobial activity of the synthesized AgNPs was determined by an agar-diffusion method (Clinical and Laboratory Standards Institute, 2016) as described for the antibacterial susceptibility testing of the crude extract and fractions above. Control plates which include organism viability control (OVC), medium sterility control (MSC), silver nitrate solution sterility control (SSC) and extract sterility control (ESC) were made in parallel and observed. The antibacterial activity of AgNPs was compared with that of the Amoxicillinand Clavulanate Potassium (0.05 mg/mL). The negative control plate was also observed.

Determination of minimum inhibitory concentration of the synthesized silver nanoparticles
The minimum inhibitory concentration (MIC) of the synthesized silver nanoparticle (AgNP) was determined against the test organisms according to the method of Omeje and Kelechi (2019). The concentrations of the synthesized AgNPs were varied (200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.3 mg/mL and 3.13 mg/mL). The test tubes containing varying concentration of agent were inoculated with a loopful of the standardized test organism using a sterile wire loop. Incubation was done at 37 ℃ for 24 h. The MIC of the extract was determined by observing the lowest concentration of the extract that inhibited growth of each organism. The MBC of the agent was also determined.

Determination of minimum bactericidal concentration
The method of Omeje and Kelechi (2019) was employed for the MBC determination. A loopful of content of MIC tube above was inoculated into fresh sterile nutrient agar plate and incubated at 37 ℃ for 24 h. Absence of growth was interpreted as MBC while presence of growth was interpreted as bacteriostatic.

Statistical analysis and data evaluation
All numeric data generated were expressed as the mean ± standard error of mean (SEM). Comparison between different groups was performed using analysis of variance (ANOVA Test). The significant differences between control and experimental groups were assessed using Duncan's multiple range test (DMRT) using SPSS version 19 at 95% level of significance.

Results
Description of *Eudrilus eugeniae* extract and fractions
Table 1 shows the physical appearance and percentage recovery of *Eudrilus eugenia* crude ethanol extract (E), ethyl acetate (EC), n-hexane (NH), and aqueous (A) fractions. Aqueous fraction had the highest percentage recovery (51.56 %) followed by ethanol crude extract (21 %), ethyl acetate extract (19.69 %) while n-hexane extract had the least percentage recovery (14.38 %).

Table 1. Physical characteristics of crude extract and fractions of *Eudriluseugeniae*

Extract/fractions	Code	Colour	Appearance	Weight (g)	% Recovery
Ethanol	E	Deep brown	Gummy mix	32	21
Ethylacetate	EC	Deep brown	Gummy mix	6.3	19.69
N-Hexane	NH	Brown	Sticky	4.6	14.38
Aqueous	A	Brown	Sticky	16.5	51.56

*E: Ethanol extract; EC:Ethylacetate fraction; NH: N-hexane fraction;A: Aqueous residual fraction; %: Percentage.

$$\text{Percentage recovery} = \frac{\text{weight of the extract obtained}}{\text{Weight of the pulverized } E.\ eugeniae} \times \frac{100}{1} \quad (1)$$

Chemical composition of crude *Eudrilus eugeniae* extract and fractions

Qualitative chemical components of crude extract and fractions of *Eudrilus eugeniae*

The chemical components present in the crude ethanol extract (E), ethyl acetate (EC), n-hexane (NH) and aqueous (A) fractions are shown in Table 2. Steroids and proteins were present in all the extract and fractions of *E. eugeniae* while alkaloids, tannins and flavonoids were absent. Saponins were present in crude ethanol and aqueous fraction while phenols were present only in aqueous fraction. Also, terpenoids were present only in ethanol extract.

Quantitative chemical components of *Eudrilus eugeniae*

The quantitative chemical composition of *E. eugeniae* is shown in Table 3. The results revealed higher concentrations of cyanides (2975.800), phytates (2471.36), moderate amount of phenols (158.084), saponins (100.992) and very low amount of oxalates (0.3955).

Table 2: Qualitative chemical components of *Eudrilus eugeniae*

Chemical components	Extracts			
	E	EC	NH	A
Saponins	+	-	-	+
Alkaloids	-	-	-	-
Phenols	-	-	-	+
Tannins	-	-	-	-
Flavonoids	-	-	-	-
Steroids	+	+	+	+
Proteins	+	+	+	+
Terpenoids	+	-	-	-

- : Absent; +: Present; NH: N-hexane fraction; EC: Ethyl acetate fraction; E: Ethanol extract; A: Aqueous residual fraction.

Table 3: Quantitative chemical components of *Eudrilus eugeniae*

Components	Quantity (mg/100g)
Phenols	158.084
Flavonoids	20.986
Alkaloids	19.198
Cyanide	2975.800
Phytates	2471.364
Oxalates	0.3955
Tannins	21.110
Saponins	100.992

mg: milligram.

Morphological characteristics and identities of bacterial isolates

The identities of the test organisms are summarized in Table 4. The organisms isolated were *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumoniae and Pseudomonas aeruginosa.*

Antibacterial activity of crude *E. Eugeniae* extract and fractions against test isolates

The antibacterial activity of the crude *E. eugeniae* extract and fractions against test isolates at 100mg/ml are shown in Table 5. The zones of inhibition produced by ethanol extract were between 1.67±1.52 mm and 3.00±1.00 mm against *Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pyogenes*, *Staphylococcus aureus* and *Escherichia coli* respectively while that of ethyl acetate fraction were between 1.33±0.58 mm and 3.67±0.58 mm against *K. pneumonia, S. aureus*, *S. pyogenes*, *E. coli* and *S. aureus* respectively. The zones of inhibition produced by aqueous residual fraction were between 1.00±1.00 mm and 5.00±1.00 mm against *K. pneumoniae, S. pyogenes, S. aureus*, *E. coli* respectively. The n-hexane (NH) fraction zones of inhibition were between 0.33±0.58 mm and 2.33±0.58 mm against *P. aeruginosa, S. pyogenes*, *S. aureus* and *Escherichia coli* respectively.

Characteristics of *Eudrilus eugeniae* extract mediated silver nanoparticles

Silver nanoparticles in the solution appeared as dark brown colour. The UV-visible spectrum of biosynthesized silver nanoparticle (AgNPs) are shown in Figure 1 and indicated the absorbance peak at 408 nm. The SEM micrograph of the particle (Fig. 2) showed spherical and less aggregated morphology with size ranging from 10-50 nm. The EDX (Fig. 3) showed the elemental composition of the particle and their atomic numbers while the XRD (Fig. 4) displayed the crystalline nature of the AgNPs. The FTIR (Fig. 5) showed the functional groups present in the extract. The transmission peaks of the particles were at 3488, 2922, 2858, 1736, 1640.1461, 1349, 1297, 1248, 1092, 946, 846 and 723 cm^{-1} respectively. The distinct peak at 2858 cm^{-1} corresponded to the bonding vibration of alkanes (C-H) while the peak at 1736cm^{-1} is an indicative of C=O stretch of esters. Furthermore, the band at 1092 cm^{-1} resulted from the C-O bending vibration of alcohol functional group.

Table 4 : Morphological and biochemical identities of isolates

GR	S	OX	CG	CT	IN	MR	VP	CI	UR	H_2S	Suger Fermentation				Haemolysis			Suspected Organism
											L	S	G	F	α	β	γ	
G-	Rod	--	--	+	+	+	--	--	--	--	+	+	+	--	--	--	--	*Escherichia coli*
G+	Cocci	--	+	+	--	+	+	+	+	--	+	+	+	+	--	+	--	*Staphylococcus aureus*
G+	Cocci	--	--	--	+	+	--	--	--	--	+	+	+	+	--	+	--	*Streptococcus pyogenes*
G-	Rod	--	--	+	--	--	+	+	+	--	+	+	+	+	--	--	--	*Klebsiella pneumoniae*
G-	Rod	+	--	+	--	--	--	+	--	--	--	--	--	--	--	--	+	*Pseudomonas aeruginosa*

*GR: Gram's reaction, S: Shape, G+: Gram positive, G-: Gram negative, OX: Oxidase, CG: Coagulase, CT: Catalase, IN: Indole, MR: Methyl red, Vogues-Proskauer, CI: Citrate utilization, UR: Urease, H_2S: Hydrogen sulphide production, , L: Lactose sugar fermentation, S: Sucrose sugar fermentation, G: Glucose sugar fermentation, F: Fructose sugar fermentation, α: Alpha haemolysis production, β: Beta heamolysis production, γ: Gamma haemolysis production.

Table 5:Antibacterial activity of *Eudrilus eugeniae crude* extract and fractions at 100 mg/ml

Extract	*Escherichia coli*	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
E	3.00 ± 1.00^{ab}	2.67 ± 0.58^{a}	2.33 ± 2.08^{a}	2.33 ± 1.53^{a}	1.67 ± 1.53^{a}
EC	3.00 ± 1.00^{ab}	3.67 ± 0.58^{ab}	3.33 ± 0.58^{ab}	2.33 ± 0.58^{a}	1.33 ± 0.58^{a}
A	5.00 ± 1.00^{a}	2.33 ± 0.58^{a}	2.00 ± 1.00^{a}	2.33 ± 1.53^{a}	1.00 ± 1.00^{a}
NH	2.33 ± 0.58^{b}	0.67 ± 0.57^{b}	0.33 ± 0.58^{b}	0.33 ± 0.58^{ab}	0.00 ± 0.00^{ab}
Control	20.00 ± 1.00^{c}	21.00 ± 1.50^{c}	21.00 ± 1.50^{c}	20.00 ± 1.10^{c}	20.00 ± 0.00^{b}

Values are zones of inhibition mean ± standard error of mean of triplicate determinations. Values with the same superscript in the same column are not significantly different at $p\leq0.05$. E: Ethanol extract, EC: Ethyl acetate fraction, A: aqueous residual fraction, NH: N-hexane fraction, control: Amoxicillin and Clavulanate Potassium, mg/ml: milligram per milliliter.

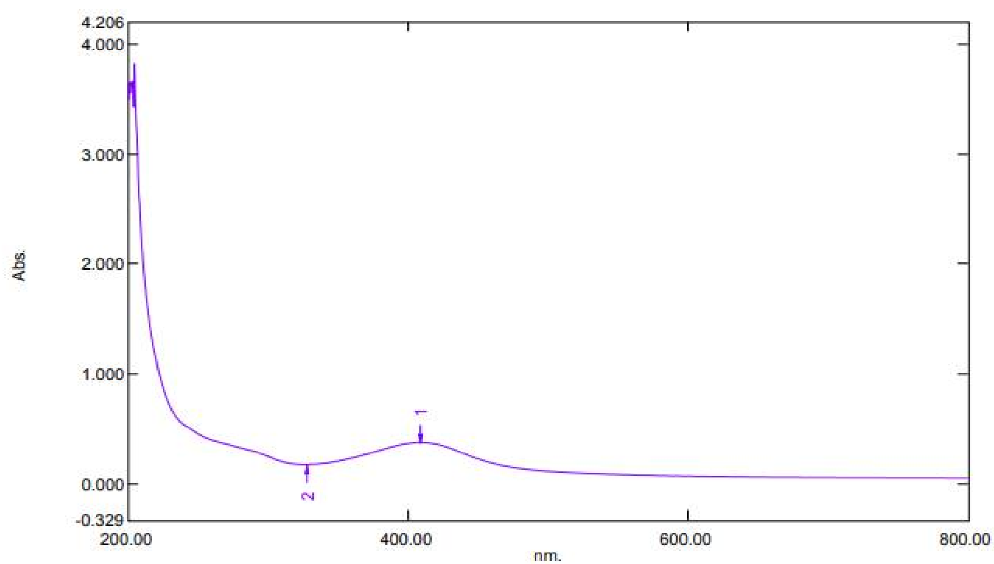

Fig. 1: Ultra Violet-Visible spectrum of aqueous residual fraction mediated Silver Nanoparticles

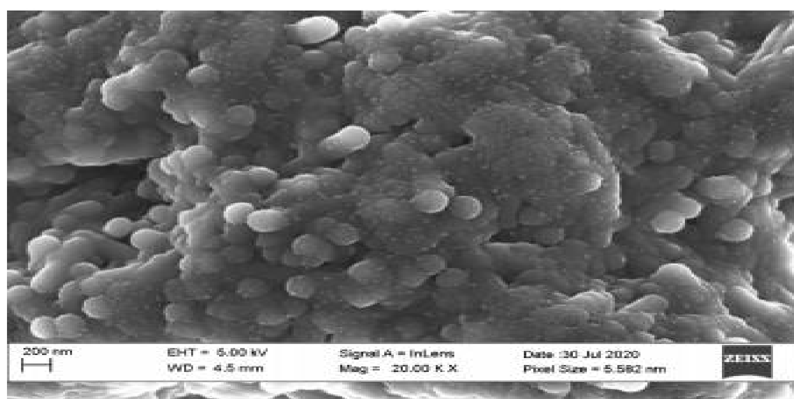

Fig. 2: Scanning electron micrograph of *Eudrilus eugeniae* aqueous residual fraction mediated silver nanoparticles

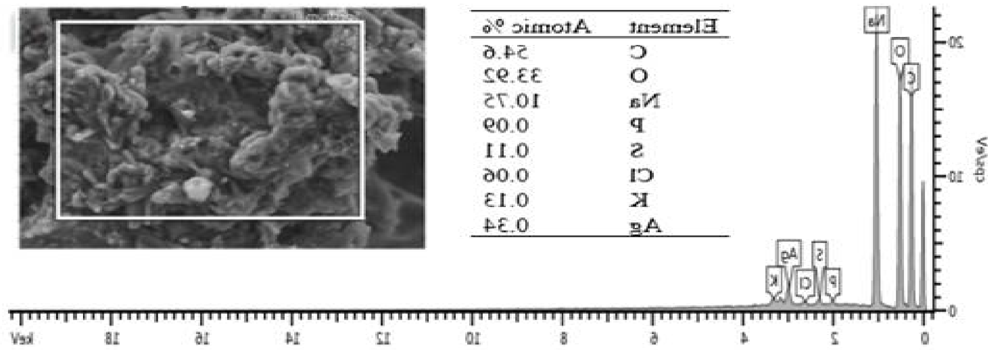

Fig. 3: Energy dispersive pattern of *Eudrilus eugeniae* aqueous residual fraction mediated silver nanoparticles

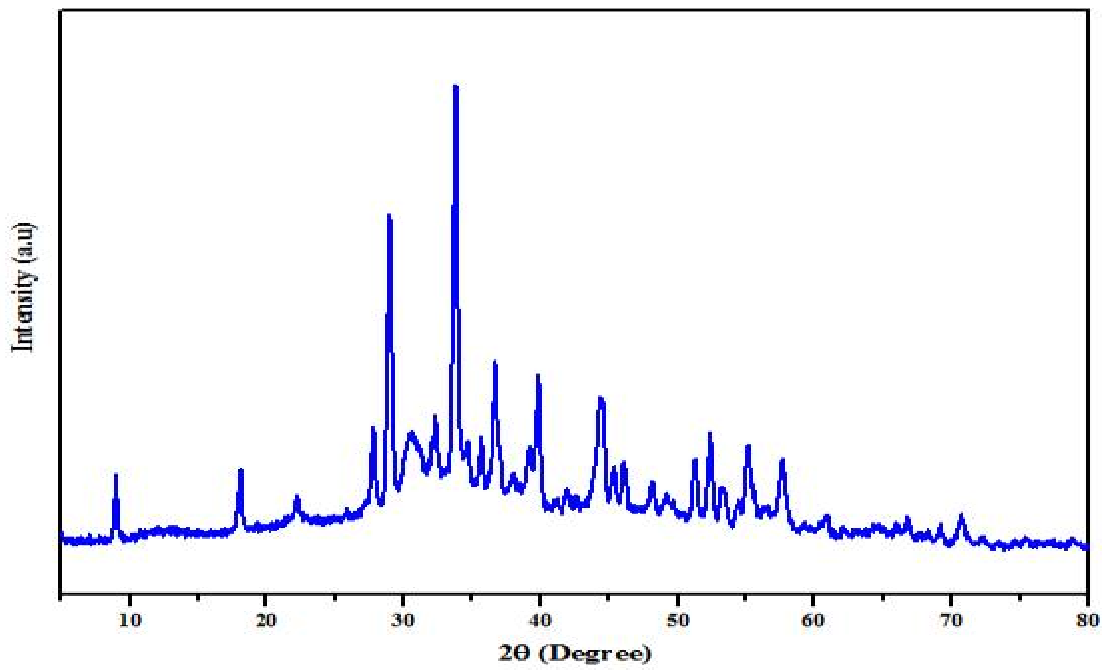

Fig. 4: X-ray diffraction pattern of *Eudrilus eugeniae* aqueous residual fraction mediated silver nanoparticles

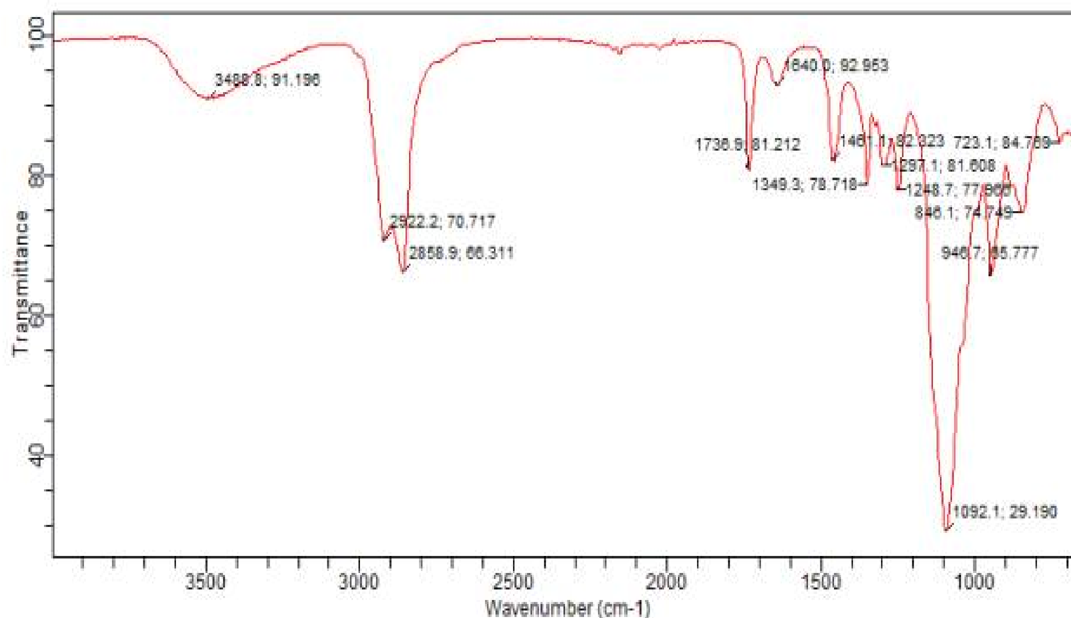

Fig. 5: Fourier-transform infrared spectrum of aqueous residual fraction mediated silver nanoparticles

Antibacterial activity of *Eudrilus eugeniae crude* extract, fractions and extract mediated silver nanoparticles against test isolates

Table 6 shows the antibacterial activity of *Eudrilus eugeniae* crude extract and fractions against test isolates. The zones of inhibition produced by crude ethanol extract were between 0.33±0.58 mm and 2.00±1.00 mm against *S. pyogenes*, *K. pneumonia*e, *S. aureus* and *E. coli.* Zones of inhibition produced by ethyl acetate fraction were between 1.00±1.00 mm and 3.33±1.16 mm against *S. pyogenes, E. coli, P. aeruginosa, K. pneumonia*e and *S. aureus* while the zones of inhibition produced by aqueous residual fraction were 0.67±1.16 mm and 2.67±1.53 mm against *S. pyogenes, K. pneumoniae*, *S. aureus, P. aeruginosa,* and *E. coli.* Zone of inhibition produced by n-hexane was 0.67±0.58 mm against *S. aureus*. The antibacterial activity of *Eudrilus eugeniae* aqueous residual fraction mediated silver nanoparticles against test isolates is shown in Table 6. The zones of inhibition produced by the synthesized AgNPs (at 1:4 dilution) were between 6.00±1.00 mm and 17.67±0.58 mm against *Streptococcus pyogenes, Staphylocccus aureus, Klebsiella pneumoniae, Escherichia coli and Psuedomonas aeruginosa* respectively while at 1:5 dilution, the zones of inhibition were between 7.67±1.52mm and 15.33.±1.16mm against *S. aureus,, S. pyogenes E. coli, K. pneumoniae and P. aeruginosa* respectively.

Minimum inhibitory concentration of synthesized silver nanoparticles against isolates

The minimum inhibitory concentration (MIC) of synthesized silver nanoparticles against test organisms is shown in Table 7.The MIC of the synthesized nanoparticles against *P. aeruginosa, S. pyrogenes* and *S. aureus* were at 6.12 mg/mL, 25 mg/mL and 50 mg/mL respectively while for *E. coli* and *K. pneumonia*e was at 25 mg/Ml.

Minimum bactericidal concentration
The synthesized silver nanoparticles was bacteriostatic on the isolates.

Table 6: Antibacterial activity of *Eudrilus eugeniae crude* extract, fractions and extract mediated silver nanoparticles at 200 mg/mL

Extract	*Escherichia coli*	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
E	2.00 ± 1.00^{a}	2.00 ± 1.00^{a}	0.33 ± 0.58^{a}	0.00 ± 0.00^{a}	1.33 ± 1.16^{a}
EC	2.00 ± 1.00^{a}	3.33 ± 1.16^{ab}	1.00 ± 1.00^{ab}	2.67 ± 0.58^{ab}	2.67 ± 1.53^{ab}
A	2.67 ± 1.53^{a}	2.00 ± 1.00^{a}	0.67 ± 1.16^{a}	2.00 ± 1.73^{ab}	1.00 ± 1.00^{a}
NH	0.00 ± 0.00^{ab}	0.67 ± 0.58^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
(AF1:4 $AgNO_3$)	15.33 ± 0.58^{b}	7.33 ± 1.53^{c}	6.00 ± 1.00^{c}	17.67 ± 0.58^{b}	7.667 ± 1.53^{b}
(AF1:5AgNO3)	12.33 ± 2.08^{c}	7.67 ± 1.52^{c}	9.33 ± 2.52^{b}	15.33 ± 1.16^{c}	14.67 ± 0.58^{c}
Control	20.00 ± 1.00^{d}	21.00 ± 1.50^{d}	21.00 ± 1.50^{d}	20.00 ± 1.10^{d}	20.00 ± 0.00^{d}

Values are zones of inhibition mean ± standard error of mean of triplicate determinations. Values with the same superscript in the same column are not significantly different at $p\leq0.05$. E; Ethanol extract, EC: Ethyl acetate fraction, A: aqueous residual fraction, NH: N-hexane, mg/ml: milligram per milliliter, AF1:4 $AgNO_3$: 1 ml of aqueous residual fraction to 4 ml of silver nitrate, AF1:5 $AgNO_3$: 1 ml of aqueous residual fraction to 5 ml of silver nitrate, control: Amoxicillin and Clavulanate Potassium (0.05 mg/mL).

Table 7: Minimum inhibitory concentration of synthesized silver nanoparticles

Isolates	Concentrations (mg/mL) 100	50	25	12.5	6.12	3.125	MIC
Escherichia coli	+	+	+	+	-	-	12.5
Staphylococcus aureus	+	+	-	-	-	-	50
Streptococcus pyogenes	+	+	+	-	-	-	25
Pseudomonas aeruginosa	+	+	+	+	+	-	6.12
Klebsiella pneumoniae	+	+	+	+	-	-	12.5

+: Activity, -: No activity, mg/ml: milligram per milliliter, MIC- Minimum inhibitory concentration

Discussion

Saponins, phenols, steroids, proteins and terpenoids were present in the crude extract and fractions of *E. eugeniae*. The results coincided with the findings of Mizanur *et al*. (2016), Justin and Ndjouka (2017) on species of *Amorphophallus* and *Lophira* containing alkaloids, saponins, flavonoids, steroids and similar components. In the present study, high concentration of cyanides (2975.800) and phytates (2471.364) were observed in dried *Eudrilus eugeniae.* Zhou *et al.* (2019) reported that phytates inhibit the growth of microorganisms by damaging their cell membrane. According to Abishek *et al*. (2020), cyanides in combination with hydrogen inhibit the growth of microorganisms. The high concentration of cyanides and phytates in earthworm may be useful for its defense and protection against pathogens in the soil.

In the present study, the isolates identified were *Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* Similar organisms were identified by Muhammad *et al*. (2020) using same biochemical tests. Crude ethanol extract (E), ethylacetate (EH) and aqueous (A) fractions of *E. eugeniae* at 100 mg/mL and 200 mg/mL respectively displayed very weak inhibitory effect on the test organisms. The n-hexane fraction was unable to inhibit the growth of all the test isolates at 200 mg/ml. The weak activity of the extracts could be as a result of low concentration of antibacterial components in the extracts and extraction capacity of the solvents. This is in agreement with the report of Ashraf and Bakri (2018).

The synthesis of silver nanoparticles using *E. eugeniae* aqueous (A) fraction was accompanied by colour change from yellow to dark brown in aqueous solution due to excitation of surface plasmon resonance (Adelere *et al.,* 2017). Similarly, Jaganathan *et al.* (2016) and Alsaggaf (2021) reported that the silver nanoparticles exhibited colour change from yellow to dark brown. The maximum absorbance peak for the synthesized silver nanoparticles (AgNPs) was observed at 408 nm which is within the range of 391 to 460 nm absorbance characteristics of silver nanoparticles earlier reported by Adelere *et al.* (2017). Concentration and pH that had been identified as factors affecting the yield of silver nanoparticles were optimized. The scanning electron (SEM) micrograph showed spherical and less aggregated morphology of nanoparticles ranging from 10 to 50 nm. Adelere *et al*. (2017) reported the spherical shape of silver nanoparticles while Lateef *et al.* (2016) reported the synthesis of spherical AgNps in the size range of 3 to50 nm.

Fourier transform infrared (FTIR) pattern confirmed the bioreduction of Ag^+ ions to silver nanoparticle which may be due to the reduction by capping material of *E. eugeniae* aqueous extract and revealed the presence of biomolecules which include alkanes, alcohol and ester functional groups. Similarly, Hossam *et al.* (2015) reported that earthworm is rich in protein which can bind to the silver nanoparticles through amino or carboxylic groups. In this finding, energy dispersive x-ray (EDX) spectrum exhibited signals of silver and other elements from the synthesized nanoparticles. The EDX spectrum indicated weak signals of chlorine, silver, phosphorus, sulphur and potassium. This may be due to the biomolecules in the extract binding to the surface of biosynthesized AgNPs. However, the high presence of signal of carbon, oxygen and sodium may be due to environmental interference during sample preparation on a glass substrate. This finding contradicts that of Roua and Sabah (2021) who reported high presence of silver with moderate amount of carbon and chlorine.

The XRD peaks were observed at 2θ (30°, 35^0, 37^0,40^0,45^0,50^0,52^0,55^0 and 58^0) respectively which indicated the crystalline nature of the AgNPs. These findings is similar to the observation by Mehta *et al.* (2017) who reported the strong diffraction peaks of AgNps at 2θ value of 32.35^0, 38.29^0, 46.38^0, 54.66^0 and 64.82^0 respectively. The sharpness of the diffraction peaks indicated the crystalline nature of nanoparticles. Silver nanoparticles obtained from *E. eugeniae* aqueous extract was able to inhibit the growth of *K. pneumoniae, E. coli* and *P aeruginosa* significantly ($p \leq 0.05$) as against the crude counterpart. This result is in line with the study of Swarnali *et al.* (2020) who reported the antimicrobial effects of biosynthesized silver nanoparticles on Gram negative and Gram positive bacteria. The MIC of the synthesized AgNPs against *P. aeruginosa* was at 6.12 mg/mL while it was at 12.5 mg/mL for *K. pneumoniae* and *E. coli*. The extract showed bacteriostatic effect against the test isolates.

Conclusion

The overall results of this study provide supportive data on the use of AgNPs from *E. eugeniae* for the treatment of diabetic wound infection.

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