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Synthesis of Gold Nanoparticles Using Bioreductor from Cocoa (*Theobroma cacao* L.) Rind Extract and Its Antibacterial Activity Test

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Abstract. This study explores the synthesis of gold nanoparticles (AuNPs) using a bioreductor from cocoa (*Theobroma cacao* L.) rind extract and evaluates their antibacterial activity. The green synthesis method employed methanol to extract organic compounds from the cocoa rind, which act as bioreductants. The formation of AuNPs was observed after 30 minutes of stirring. Characterization was performed using UV-Vis spectrophotometry, Fourier Transform Infrared (FTIR) spectroscopy, X-Ray Diffraction (XRD), and Scanning Electron Microscopy-Energy Dispersive X-Ray (SEM-EDX). UV-Vis spectrophotometry revealed a maximum absorbance at 553 nm. FTIR analysis indicated the presence of hydroxyl groups acting as reducing agents. XRD results confirmed the Face Centered Cubic (FCC) structure of the AuNPs, with an average diameter of 6.303 nm as determined by the Scherrer equation. SEM-EDX analysis showed spherical nanoparticles. The antibacterial activity was tested against *Pseudomonas aeruginosa* and *Bacillus subtilis*, with inhibition zones of 8.9 mm and 7.6 mm, respectively. This environmentally friendly synthesis method offers a cost-effective approach to producing AuNPs with significant antibacterial properties

Introduction

Nowadays, technological developments are experiencing rapid changes and exciting innovations, both in production, size, and shape. This development can take the form of synthesis, design, and material or device applications at the nanoscale, known as nanotechnology (Patel et al., 2021). In nanotechnology, most activities focus on the synthesis of new nanoparticles in different shapes, sizes, and bioactivity effects that can be applied in various fields (Y. Khan et al., 2022). These nanoparticles are known for their multifunctional applications, including in nutrition, energy, and as supporting active substances in food and drugs to regulate the speed of release of active compounds, increase solubility, and enhance absorption in the human body (Chen et al., 2023).

Nanoparticles, characterized by their size of 1-100 nm, are solid colloidal particles that contain macromolecular material and possess unique properties, such as small size and large surface area. The specific characteristics of nanoparticles depend on the size, distribution, and morphology of the particles (K. U. Khan et al., 2022). Several factors can affect particle size in synthesis, including solute temperature, salt concentration, reducing agents, and reaction time (Ali et al., 2017).

Gold nanoparticles are one of the most widely synthesized metal nanoparticles in their ionic form due to their free radical absorber activity, which can prevent damage in various cells, such as aging and cell damage. Therefore, AuNPs are commonly applied as biosensors (Hua et al., 2021), antibacterial agents (Su et al., 2020), antioxidants (El-Borady et al., 2020), and anticancer agents (Babaei et al., 2021).

The synthesis of AuNPs can be achieved through various chemical methods such as photochemistry and chemical reduction. However, the use of radiation rays in photochemical methods can cause side effects and is less practical (De Freitas et al., 2018). Green synthesis offers a solution that can overcome the shortcomings of physical and chemical methods because it allows for the synthesis of nanoparticles at low cost, in an environmentally friendly manner, on a large scale, without requiring high pressure

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and temperature, and without using hazardous materials (Dikshit et al., 2021; Ying et al., 2022).

Some types of plants contain secondary metabolite compounds like flavonoids that can reduce substances to nanoparticle size, one of them being cocoa (*Theobroma cacao* L.) (Campos-Vega et al., 2018). Cocoa is known to contain active flavonoids and condensed or polymerized tannins such as anthocyanidins, catechins, and leukoanthocyanidins (Younes et al., 2023). These bioactive compounds have antibacterial properties and act as natural bioreductors. Phytochemical analysis has shown positive contents of alkaloids, flavonoids, tannins, and saponins from cocoa, especially in its rind. These compounds are classified as phenol compounds that act as natural reducing agents and antibacterials (Rachmawaty et al., 2018a; Younes et al., 2023).

This study investigates the innovative use of cocoa rind extract as a bioreductor for the green synthesis of AuNPs. The primary objective is to characterize the synthesized AuNPs and evaluate their antibacterial activity against specific bacteria. By utilizing agricultural waste materials, this research aims to provide a sustainable and eco-friendly method for producing AuNPs.

Experimental

Material and Methods

Cocoa rind, aquaregia, pure gold metal, methanol p.a (merck), $FeCl_3$, HCl (merck), Dragendorff reagent, Chloramphenicol, KLT plate, ethyl acetate (merck), bacterial culture of *Pseudomonas aeruginosa* and *Bacillus subtilis*, disc paper, Nutrient Broth (NB), Mueller Hinton Agar (MHA), Whatman filter paper no.41.

Fourier Transform Infra Red (FTIR), UV-Vis spectrophotometer, Scanning Electron Microscopy-Energy Dispersive X-Ray (SEM-EDX), X-Ray Diffraction (XRD), freeze dryer, autoclave, hot plate, analytical balance, magnetic stirrer and commonly used glassware in the laboratory.

Procedures

Preparation of Cocoa Rind Extract

The collected cocoa rind was cut into small pieces, washed to remove dirt, and then dried outdoors for about a week. Afterward, the drying process continued in an oven at 55 °C for about 24 hours to ensure the sample was completely dry, then it was ground into a fine powder. Two hundred grams of cocoa rind powder were soaked with methanol p.a in a 1000 mL beaker glass until the powder was submerged. Soaking was done at room temperature for 7×24 hours. Filtration was done with Whatman filter paper no.41 until the macerate was obtained. The solvent in the macerate was evaporated using a rotary evaporator until a concentrated extract was obtained. The methanol extracts obtained were subjected to phytochemical profiling, FTIR analysis, and antibacterial activity testing (Arif et al., 2022; Iryanti Eka Suprihatin & Najwa Kusuma Putri Antariksa, 2023; Rachmawaty et al., 2018b).

Media

Testing weighing MRSA media as much as 17.05 g, $CaCO_3$ as much as 2.5 g then dissolved with aquades as much as 250 mL. Then all the media are mixed and then stirred until the granules of the media dissolve into the distilled water into one. After the solution was homogeneous, the media was sterilized in an autoclave for 20 minutes at a temperature of 121 °C. After sterilization, the media was cooled until warm and poured into a 10 mL petri dish as an isolation medium. As for the purification media, we used a test tube with a media volume of 7 mL, which is used as an inclined medium.

Preparation of $HAuCl_4$ Solution

One gram of pure gold metal was dissolved in 8 mL of aqua regia (a mixture of concentrated HCl and concentrated HNO_3 in a 3:1 ratio). The solution was then transferred into a 1000 mL volumetric flask, and deionized water was added up to the calibration mark and homogenized. From this 5 mM $HAuCl_4$ stock solution, further solutions with concentrations of 0.5 mM, 1.0 mM, and 1.5 mM were prepared. (Nguyen et al., 2010).

Synthesis of Gold Nanoparticles

Determination The synthesis of AuNPs was done by mixing 40 mL of $HAuCl_4$ solution with 1 mL of cocoa rind extract in a 250 mL Erlenmeyer flask, and then stirring with a magnetic stirrer during 30 minutes at 37 °C. The solution was characterized by a color change from yellow to purple and analyzed using a UV-Vis spectrophotometer at a wavelength of 500-600 nm to confirm the formation of nanoparticles. The nanoparticles were then allowed to settle for about 24 hours to separate the filtrate from the precipitate, which was then decanted to collect the precipitate. The precipitate was dried using a freeze dryer for about 48 hours and characterized by XRD, FTIR, and SEM-EDX (Castillo-Henríquez et al., 2020; Elia et al., 2014).

Antibacterial Activity Test

The antibacterial activity of the synthesized AuNPs was tested using the paper disc diffusion method. Bacterial cultures of Pseudomonas aeruginosa and Bacillus subtilis were used in this study. The discs were prepared by soaking them in the nanoparticle solution and then placing them on Mueller Hinton Agar plates inoculated with the bacterial cultures. Chloramphenicol served as a positive control. The plates were incubated at 37 °C for 24 hours, and the inhibition zones were measured to evaluate the antibacterial efficacy of the nanoparticles (Gouyau et al., 2021).

Result and Discussion

Phytochemical Test of Methanol Extract of Cocoa Rind (*Theobroma cacao* L.)

The phytochemical analysis of the methanol extract of cocoa rind, as presented in Table 1, indicates the presence of various secondary metabolites. The table reveals positive results for alkaloids (using Dragendorff reagent), flavonoids (using $Pb(CH_3COO)_2$ reagent), phenolics, and saponins, while Meyer reagent for alkaloids, Mg reagent for flavonoids, and tests for terpenoids returned negative results.

Table 1. Phytochemical test result of methanol extract of cocoa rind.

Secondary Metabolites		Result
Alkaloid	Dragendorff reagent	+
	Meyer reagent	-
Flavonoid	$Pb(CH_3COO)_2$ reagent	+
	Mg reagent	-
Terpenoid		-
Phenolic		+
Saponin		+

The detection of these metabolites suggests a rich phytochemical profile of the cocoa rind, which is significant for its potential applications in various biochemical processes. The presence of these phytochemicals aligns with findings from existing literature on cocoa by-products. The identification of flavonoids, phenolics, and saponins in the methanol extract of cocoa rind is particularly significant for their role in the bioreduction process during the synthesis of AuNPs (Rebollo-Hernanz et al., 2022). These compounds are known for their antioxidant properties, which are crucial for reducing metal ions to form nanoparticles.

To explore the potential of methanol extract of cocoa rind as a bioreductor of nanoparticles, it is crucial to analyze the phytochemical composition of the extract. Phytochemical analysis is essential for identifying the bioactive compounds present in natural products like cocoa rind (Arif et al., 2022). Research indicates that methanol extracts from plant materials can indeed contain various phytochemicals such as flavonoids, tannins, phenols, triterpenoids, alkaloids, and other compounds (Nea et al., 2021). These phytochemicals are recognized for their antioxidant properties, which could be advantageous in reducing nanoparticles (Balkrishna et al., 2021).

Synthesis of Gold Nanoparticles

The synthesis of AuNPs, as shown in Figure 1, began with the preparation of a 5 mM $HAuCl_4$ solution, which was then concentrated to 0.5, 1, and 1.5 mM. Gold nanoparticles were formed by mixing 40 mL of the $HAuCl_4$ solution with 1 mL of cocoa rind extract, followed by stirring with a magnetic stirrer for approximately 30 minutes until the color changed from yellow to purple. This color change indicates the successful formation of nanoparticles. The color shift occurs due to the Surface Plasmon Resonance (SPR) excitation of AuNPs, indicating the reduction of Au^{3+} metal ions to Au^0, facilitated by compounds in the cocoa rind extract. This transformation can be confirmed by measuring the maximum wavelength of 500-600 nm using a UV-Vis spectrophotometer. The results can be seen in Figure 1.

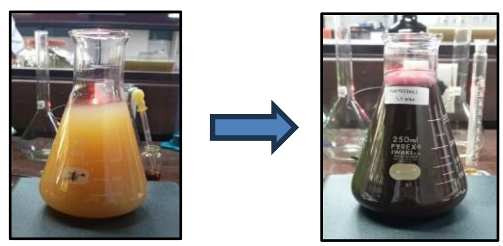


Figure 1. Synthesis of Au nanoparticles at 37 °C for 24 hours using cocoa methanol extract.

The color change resulting from the SPR excitation of AuNPs signifies the reduction of Au^{3+} metal ions to Au^0 by compounds in the cocoa rind extract. Secondary metabolite compounds play a crucial role in the formation of AuNPs. The Au^{3+} ions can form intermediate complex compounds with free radicals in flavonoid compounds, which are then oxidized while Au^{3+} is reduced to AuNPs. This green synthesis method, utilizing plant extracts, aligns with the broader trend of eco-friendly nanoparticle production, offering an efficient and sustainable approach (Saqr et al., 2021; Yuanita et al., 2022).

Characterisation of Gold Nanoparticles Using UV-Vis Spectrophotometer

The UV-Vis spectrophotometer results, as shown in Figure 2, indicate that the synthesized AuNPs exhibit typical absorbance peaks within the 500-600 nm wavelength region, confirming their formation. Specifically, at a concentration of 1.5 mM $HAuCl_4$, the nanoparticles displayed an absorbance peak at 553 nm with an absorbance value of 0.164. This peak corresponds to the Surface Plasmon Resonance (SPR) of AuNPs, which is a characteristic feature of their optical properties and signifies the successful reduction of Au^{3+} ions to Au^0.

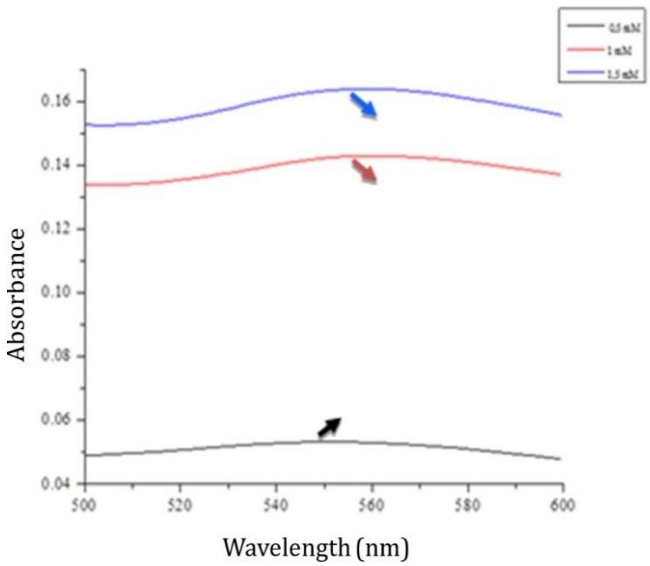


Figure 2. UV-vis spectrum of AuNPs obtained at 37 °C and 30 min reaction time.

Gold nanoparticles are effectively characterized using UV-Vis spectroscopy due to its ability to evaluate their stability and optical properties. Various studies have demonstrated the utility of UV-Vis spectroscopy in assessing the stability and properties of AuNPs synthesized from different natural sources, such as Salix alba (Islam et al., 2019), Siberian ginseng (Wu et al., 2019), clove flower water extract (Lestari et al., 2021), and goji berry (David et al., 2023). The characteristic absorption peaks observed in the UV-Vis spectra, typically between 500-600 nm, are indicative of the formation and presence of AuNPs (Lima et al., 2021; Rokkarukala et al., 2023; Ullah et al., 2024). The absorption peaks confirm the formation of AuNPs and provide insights into their stability, shape, and size (Rokkarukala et al., 2023; Ullah et al., 2024).

X-Ray Diffraction Analysis

The X-ray Diffraction (XRD) analysis of the synthesized AuNPs reveals distinct diffraction peaks at 2θ values of 38.180°, 44.191°, 64.680°, 77.646°, and 81.66°. These peaks correspond to the characteristic reflections of face-centered cubic (FCC) structures, as confirmed by the International Centre for Diffraction Data (ICDD) file No. 00784. The observed peaks align with the (111), (200), (220), (311), and (222) planes of gold, indicating that the synthesized nanoparticles possess a highly crystalline nature (Ogundare et al., 2019). The average crystal sizes calculated using the Debye-Scherrer equation ranged from 5.93 to 11.37 nm, as shown in Table 3. These results affirm the effectiveness of using methanol extract of cocoa rind in synthesizing AuNPs with controlled crystal sizes.

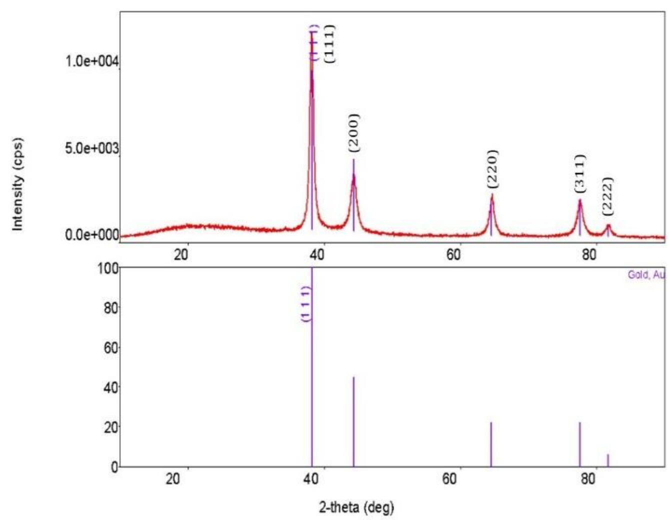


Figure 3. XRD diffractograms of AuNPs.

Comparing these results with previous studies, Wongyai similarly reported the formation of highly crystalline FCC AuNPs using plant extracts, which exhibited comparable XRD patterns (Wongyai et al., 2020). Mishra and Herrero also observed FCC structures in their XRD analyses of AuNPs synthesized through various green methods, reinforcing the consistency of these findings across different biological synthesis routes (Herrero-Calvillo et al., 2019; Mishra et al., 2022). Furthermore, Gawas demonstrated that the XRD patterns of AuNPs synthesized using biological sources revealed distinct gold facets with a mean particle size of approximately 7.18 nm, which aligns closely with the sizes obtained in this study (Gawas et al., 2023).

Table 2. Crystal size calculation of AuNPs using *Debye-Scherer* equation.

No	2θ	θ	FWHM		Size (nm)
			deg	rad	
1	38,036	19,018	0,66	0,011519	11,3797878
2	44,19	22,095	1,24	0,021642	5,93618711
3	64,4	32,2	0,71	0,012392	9,46817334

In addition, Murrieta validated the nanocrystalline nature of AuNPs by comparing their XRD spectra with standard data, confirming the reliability of XRD as a characterization technique for AuNPs. These comparative analyses highlight that the current synthesis method, utilizing methanol extract of cocoa rind, is consistent with other biological synthesis methods in producing AuNPs with desirable crystalline properties and controlled sizes (Murrieta et al., 2021).

FTIR Analysis

FTIR spectroscopy was utilized to identify the functional groups present in the methanol extract of cocoa rind and the synthesized AuNPs. Figure 4 presents the FTIR spectra, while Table 3 details the corresponding absorption data. The FTIR spectrum of the cocoa rind extract exhibits a significant absorption peak at 3419 cm^{-1}, indicating the presence of O-H groups from alcohol or phenol compounds. Strong absorption peaks at 1608 cm^{-1} and 1527 cm^{-1} correspond to aromatic C=C stretching vibrations. Additionally, medium intensity absorptions at 1257 cm^{-1} and 1053 cm^{-1} are attributed to para-substituted aromatic groups. After the formation of AuNPs, the FTIR spectrum reveals a decrease in the intensity of these functional groups, suggesting their interaction with gold ions during nanoparticle synthesis.

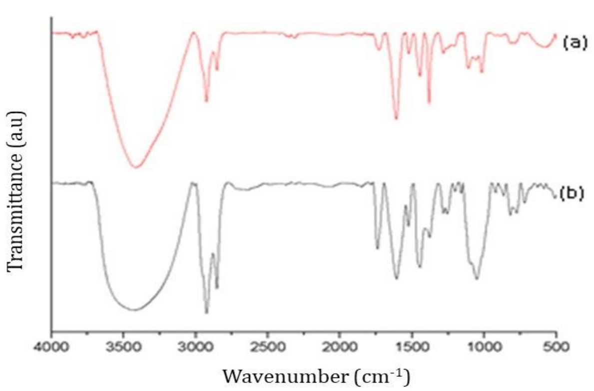


Figure 4. FTIR spectra of (a) AuNPs (b) methanol extract of cocoa rind.

The FTIR spectra provide valuable insights into the chemical interactions during the synthesis of AuNPs using the methanol extract of cocoa rind. The reduction in intensity of O-H, C-H, and C-O aryl ether functional groups observed in this study is consistent with findings from other research. For instance, Folorunso reported similar spectral changes in their studies on the characterization of bioactive compounds in cocoa extracts, indicating successful interaction and stabilization of nanoparticles (Folorunso et al., 2019).

Table 3. FTIR absorption data of cocoa rind skin methanol extract and AuNPs.

Wave number (cm^{-1})		Functional Group
Cocoa Rind Extract	AuNPs	
3419	3412	O-H
2924 and 1446	2924 and 1382	Csp^3-H
1446	1446	$-CH_2$
1446 and 1381	1446 and 1382	$-CH_3$
819	819	Para substituted aromatics
1608 and 1527	1610 and 1521	C=C aromatic
1257 and 1053	1209 and 1058	C-O aryl ether

Furthermore, Monroy utilized FTIR analysis to evaluate flavonoids and catechins in cocoa samples, highlighting the importance of this technique in determining the phytochemical profile (Monroy, 2022). In the current study, the observed decrease in functional group intensity corroborates the hypothesis that hydroxyl groups play a critical role in the reduction and stabilization of AuNPs. This is in line with findings by Ahmad, who also observed significant involvement of hydroxyl and other functional groups in nanoparticle formation using biological extracts (Ahmad et al., 2019).

The results from this FTIR analysis underscore the significance of using natural plant extracts in the green synthesis of AuNPs. The identification of functional groups involved in the synthesis process provides a deeper understanding of the mechanisms underlying nanoparticle formation. This knowledge is crucial for optimizing synthesis methods and improving the quality and consistency of nanoparticles produced.

Scanning Electron Microscopy-Energy Dispersive X-Ray (SEM-EDX) Analysis

The SEM analysis of AuNPs synthesized using cocoa rind extract revealed significant insights into their morphology. The SEM images, as shown in Figure 5, were captured at magnifications of 3000x and 5000x, providing detailed views of the nanoparticle surface. These images demonstrated that the AuNPs predominantly possess a spherical shape. The analysis was conducted with a voltage acceleration of 15 kV, ensuring high-resolution imaging of the nanoparticles at scales of 5 μm and 10 μm. This morphological information is crucial for understanding the physical characteristics of the synthesized AuNPs, particularly their size and shape distribution.

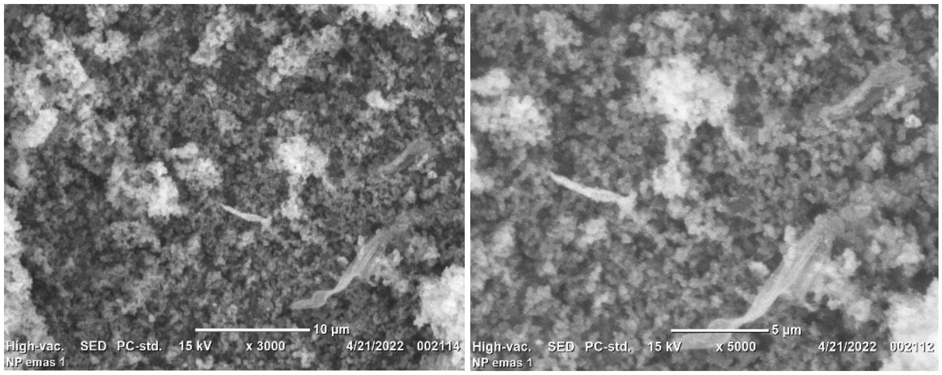
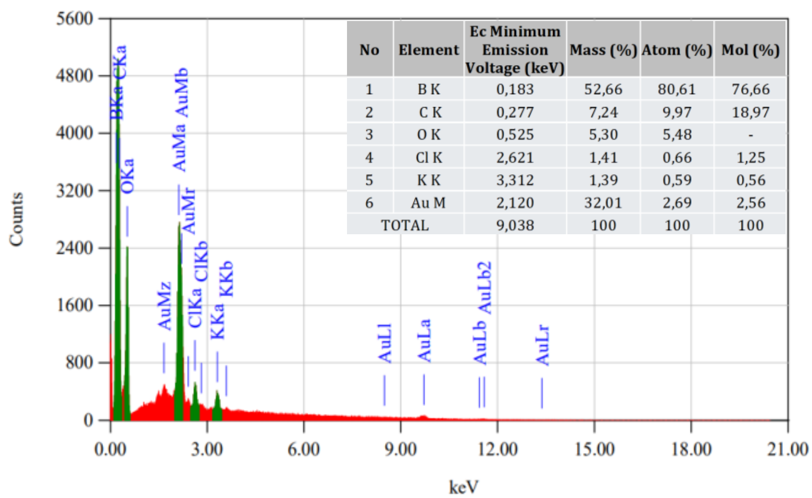


Figure 5. SEM images of AuNPs (a) 3000x and (b) 5000x.

The EDX analysis, depicted in Figure 6, was employed to determine the elemental composition of the synthesized AuNPs. This technique utilizes x-rays generated by fast electrons to provide an accurate profile of the elements present. The EDX results confirmed that the total mass of gold (Au) in the sample was 32.01%, indicating a high purity of the AuNPs. Additionally, the presence of typical optical absorption peaks between 2-4 keV confirmed the synthesis of pure AuNPs, with the SPR value shifting to lower energy visible light. The other elements detected, such as carbon (C) and oxygen (O), are likely from the secondary metabolites of the cocoa rind extract, which act as stabilizing agents.



No	Element	Ec Minimum Emission Voltage (keV)	Mass (%)	Atom (%)	Mol (%)
1	B K	0,183	52,66	80,61	76,66
2	C K	0,277	7,24	9,97	18,97
3	O K	0,525	5,30	5,48	-
4	Cl K	2,621	1,41	0,66	1,25
5	K K	3,312	1,39	0,59	0,56
6	Au M	2,120	32,01	2,69	2,56
	TOTAL	9,038	100	100	100

Figure 6. EDX profile of AuNPs.

Previous studies have demonstrated similar morphological characteristics in AuNPs synthesized using different methods. For instance, (Islam et al., 2019) observed aggregation of AuNPs with increased temperature, maintaining the typical plasmon resonance peak with a red shift. (Stojanovski et al., 2024) reported size-dependent dissolution of AuNPs when exposed to ozone, with smaller particles exhibiting higher dissolution rates. Tepale highlighted the impact of coatings on the size and morphology of green-synthesized AuNPs, showcasing spherical and homogeneous shapes.

The findings of this study align with the observations of Rotimi, who confirmed the uneven spherical shape of AuNPs with an average size of approximately 37 nm (Rotimi et al., 2019). Similarly, Rajasekar reported the presence of small, nearly spherical AuNPs through SEM analysis (Rajasekar et al., 2020). The EDX results in this study further corroborate the elemental purity observed by Gawas and Zainab, who confirmed the successful synthesis of AuNPs with high elemental composition (Gawas et al., 2023; Zainab et al., 2021).

Antibacterial activity

The antibacterial activity of AuNPs synthesized from cocoa rind extract was evaluated using the paper disc diffusion method against Pseudomonas aeruginosa and Bacillus subtilis. The results, as shown in Figure 7, demonstrated that AuNPs inhibited the growth of these bacteria, albeit to a lesser extent compared to the cocoa rind extract and the positive control, chloramphenicol. The diameter of the inhibition zones was measured after 24 hours of incubation. Table 4 summarizes the inhibition zone diameters, indicating that AuNPs produced inhibition zones of 8.9 mm against *Pseudomonas aeruginosa* and 7.6 mm against *Bacillus subtilis*.

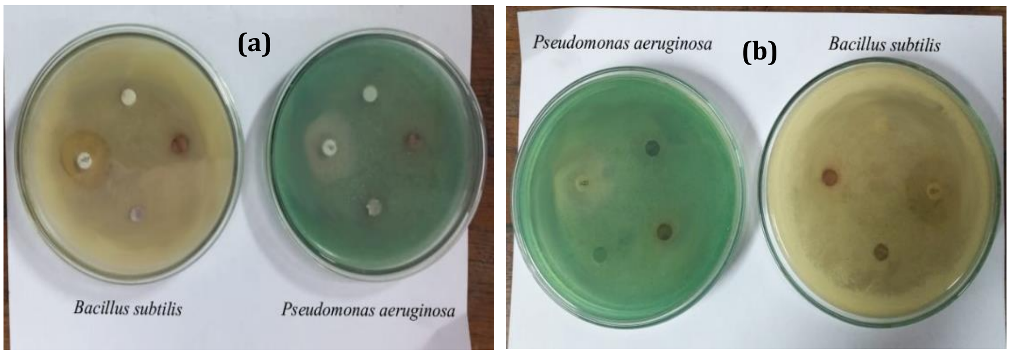


Figure 7. Antibacterial activity of AuNPs againts gram positive and gram negative organism (a) methanol extract of cocoa rind, (b) AuNPs.

The results of the antibacterial tests reveal that AuNPs exhibit moderate antibacterial activity. The inhibition zone diameters for Pseudomonas aeruginosa and *Bacillus subtilis* are 8.9 mm and 7.6 mm, respectively, as shown in Table 4. In comparison, the cocoa rind extract demonstrated higher inhibition zones of 12.3 mm for Pseudomonas aeruginosa and 11.1 mm for *Bacillus subtilis*. The positive control, chloramphenicol, showed the largest inhibition zones, with diameters of 26.5 mm and 23.3 mm, respectively. According to Cane et al, The classification of antimicrobial strength is typically based on the size of inhibition zones, with zones less than 12 mm considered weak, 12-20 mm as moderate, and over 20 mm as strong (Cane et al., 2020). Thus, the AuNPs exhibit weak inhibitory power compared to cocoa rind extract and chloramphenicol.

Table 4. Inhibition zones of tested bacterial strains against AuNPs.

Sample	Inhibition diameter (mm)	
	P. aeruginosa	*B. subtilis*
AuNPs	8,9	7,6
Methanol extract of Cocoa Rind	12,3	11,1
Positive Control	26,5	23,3
Negative Control	5,8	6

These findings align with previous studies demonstrating the antibacterial properties of AuNPs. AuNPs have been shown to possess significant antibacterial activity against both Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Bacillus subtilis*. For instance, Sreedharan and Singh found that AuNPs impregnated with antibiotics were effective against various bacterial strains (Sreedharan & Singh, 2019). Hamid and Mahmood further highlighted the enhanced antibacterial efficacy of AuNPs functionalized with antibiotics like ciprofloxacin and nisin pectin bioconjugate (Hamid & Mahmood, 2021). The ability of AuNPs to permeate bacterial cell membranes and disrupt membrane potential is a key factor in their antibacterial action.

Moreover, Essid reported that AuNPs could inhibit bacterial enzymes involved in DNA replication and protein synthesis, thereby impeding bacterial growth (Essid et al., 2023). The low toxicity, high stability, and ease of modification of AuNPs make them attractive candidates for addressing multidrug-resistant bacteria (Folorunso et al., 2019). As a conclude, the antibacterial activity of AuNPs synthesized from cocoa rind extract is evident, albeit weaker than that of cocoa rind extract and chloramphenicol. These nanoparticles have the potential to be further developed and functionalized to enhance their antibacterial efficacy, making them valuable tools in the fight against bacterial infections.

Conclusion

Based on the results of this study, it can be concluded that the methanol extract of cocoa rind (*Theobroma cacao* L.) contains compounds capable of functioning as bioreductors in the synthesis of AuNPs. The resulting nanoparticles exhibited varying wavelengths in the range of 540-560 nm. Additionally, the successfully synthesized AuNPs had an average diameter of 8.928 nm and a Face Centered Cubic (FCC) crystal structure, with a crystal diameter ranging from 5.93 to 11.37 nm. The AuNPs demonstrated the ability to inhibit the activity of Pseudomonas aeruginosa and Bacillus subtilis bacteria, with inhibition zones measuring 8.9 mm and 7.6 mm, respectively. This indicates the potential use of cocoa rind extract for eco-friendly and effective synthesis of AuNPs with antibacterial properties.

Conflict of Interest

The authors declare that there is no conflict of interest.

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