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ORIGINAL RESEARCH

Cocoa pod husk extract-mediated biosynthesis of silver nanoparticles: its antimicrobial, antioxidant and larvicidal activities

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Abstract The present investigation reports utility of cocoa pod husk extract (CPHE), an agro-waste in the biosynthesis of silver nanoparticles (AgNPs) under ambient condition. The synthesized CPHE-AgNPs were characterized by UV–visible spectroscopy, Fourier-transform infrared spectroscopy, Energy dispersive X-ray (EDX) spectroscopy and transmission electron microscopy. The feasibility of the CPHE-AgNPs as antimicrobial agent against some multidrug-resistant clinical isolates, paint additive, and their antioxidant and larvicidal activities were evaluated. CPHE-AgNPs were predominantly spherical (size range of 4–32 nm) with face-centered cubic phase and crystalline conformation pattern revealed by selected area electron diffraction, while EDX analysis showed the presence of silver as a prominent metal. The synthesized nanoparticles effectively inhibited multidrug-resistant isolates of *Klebsiella pneumonia* and *Escherichia coli* at a concentration of 40 μg/ml, and enhanced the activities of cefuroxime and ampicillin in synergistic manner at 42.9–100 % concentration, while it completely inhibited the growth of *E. coli*, *K. pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus niger* as additive in emulsion paint. The antioxidant activities of the CPHE-AgNPs were found to be excellent, while highly potent larvicidal activities against the larvae of *Anopheles* mosquito at 10–100 μg/ml concentration were observed. Our study demonstrated for the first time the utility of CPHE in the biosynthesis of CPHE-AgNPs with potential applications as antimicrobial and larvicidal agents, and paint additives for coating material surfaces to protect them against microbial growth while improving their shelf life.

Keywords CPHE-AgNPs · Antimicrobial activity · Multidrug resistance · Paint additive · Antioxidant · Larvicidal

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Introduction

Biosynthesis of nanoparticles of metals such as gold, silver, zinc, copper, platinum and palladium has received great attention from researchers all over the world. This is due to their wide application in medical and pharmaceutical fields, especially as ingredients of most consumer products like shampoo, soaps, detergents, shoes, cosmetics and toothpastes [1]. Green chemistry has become an expanded area of nanotechnology for a number of reasons which include its low cost, less demanding and eco-friendly

nature that is devoid of use of hazardous chemicals and procedures. The advent of green technology into the synthesis of nanoparticles has greatly revolutionized the field of nanotechnology. Firstly, it has opened up the possibility of using biomolecules/substances of diverse origin in its synthesis and secondly, it has widened its applicability in different areas of human endeavors. Utilization of green synthesized nanoparticles transverses medical and biomedical applications to solving environmental problems such as land and water pollution, through material engineering to applications in agriculture.

Quite a number of biological macromolecules/substances have been employed as capping and stabilizing agents for the green synthesis of nanoparticles. For instance, metabolites of some arthropods have been used for green synthesis of nanoparticles [2–5]. In several studies, metabolites and enzymes of microbial origin (fungi and bacteria), even whole microbes have been used in the synthesis of nanoparticles [6–11], while many others have reported the synthesis of nanoparticles by employing extracts from various parts of the plants such as seeds, fruits, flowers, leaves, stem and roots [12–19]. Kaviya et al. [20], Njagi et al. [21] and Roopan et al. [22] synthesized AgNPs using *Sorghum* spp bran extract, *Citrus cinensis* peel extract and *Cocos nucifera* coir, respectively, suggesting the usefulness of agro-wastes in green nanotechnology. Most recently, the usefulness of *Cola nitida* pod, seed and seed shell for the green synthesis of AgNPs has been demonstrated in our laboratory [23, 24].

Cocoa (*Theobroma cacao*) is one of the key economic crops cultivated in Nigeria. As the third largest producer of cocoa in Africa and one of the highest cocoa producer in the world, the production capacity of Nigeria was reported to have reached about 385,000 tonnes per annum on the cultivable area of 966,000 ha with an appreciable increase of about 215,000 from year 2000 production level [25, 26]. The implication of this is that as the cocoa production industry is expanding, so also the cocoa pod husk (CPH) which is the by-product of cocoa processing that account for 52 to 76 % of the cocoa pod wet weight [27]. Cocoa is the principal ingredient of chocolate and other derived products such as cocoa liquor, cocoa butter, cocoa cake and cocoa powder, whereas the abundantly produced cocoa pod husks as waste in cocoa plantations during the extraction of cocoa beans are often discarded as of no market value. However, to create valuable products, cocoa pod husks have been explored as food antioxidants [28], dietary fibers [29], animal feed [30], as a precursor in the activated carbon production [31], fertilizer [32] and thermoplastic polyurethane composites [33]. It has also been investigated for use in the production of enzyme, with resultant improvement of the nutritional quality of the husk through fungal solid substrate fermentation [34].

The need to find alternative usage for agro-wastes motivated the present investigation into the biotechnological potential of cocoa pod husk extract (CPHE) in green chemistry for the synthesis of CPHE-AgNPs. Therefore, the present study was designed to explore the utility of CPHE in the synthesis of CPHE-AgNPs, evaluation of the antioxidant activities, mosquito larvicidal activities, and antimicrobial potentials of the synthesized nanoparticles against multidrug-resistant clinical bacteria. In addition, the usefulness of the synthesized CPHE-AgNPs as antimicrobial additive in emulsion paint was demonstrated. To the best of our knowledge, this report is the first of its kind on the use of cocoa pod husk extract (CPHE) for the synthesis of CPHE-AgNPs.

Materials and methods

Collection and processing of cocoa pods

Fresh cocoa fruits were obtained from Ipetumodu, Osun State, Nigeria. They were brought to the laboratory and thoroughly washed to remove dirt and other extraneous substances. The pods were split opened with the pod husk and beans separated. The pod husks were first transformed into chips and then air dried for seven days at room temperature (30 ± 2 °C). The pod husk chips were milled into powder with the aid of electric blender (Fig. 1).

Preparation of cocoa pod husk extract

Cocoa pod husk extract (CPHE) was obtained following the methods of Lateef et al. [23] by weighing 0.1 g of the powder and suspended in 10 ml of distilled water, and heated in water bath at 60 °C for 1 h. The extract was filtered using Whatman No. 1 filter paper and then centrifuged at 4000 rpm before the collection of the final clear extract (CPHE), which was stored at 4 °C for further use.

Green synthesis of CPHE-AgNPs and characterization

The CPHE prepared was used to synthesize CPHE-AgNPs using the protocol previously described [23, 24]. To 40 ml of 1 mM silver nitrate ($AgNO_3$), 1 ml of the extract was added at room temperature (30 ± 2 °C) and the reaction mixture was allowed to stand for some minutes. A change in color of the reaction mixture was visually observed, followed by the measurement of its absorbance spectrum using UV–visible spectrophotometer (Cecil, USA) operated at the range of 200–800 nm. FTIR spectroscopy analysis was carried out on the synthesized CPHE-AgNPs

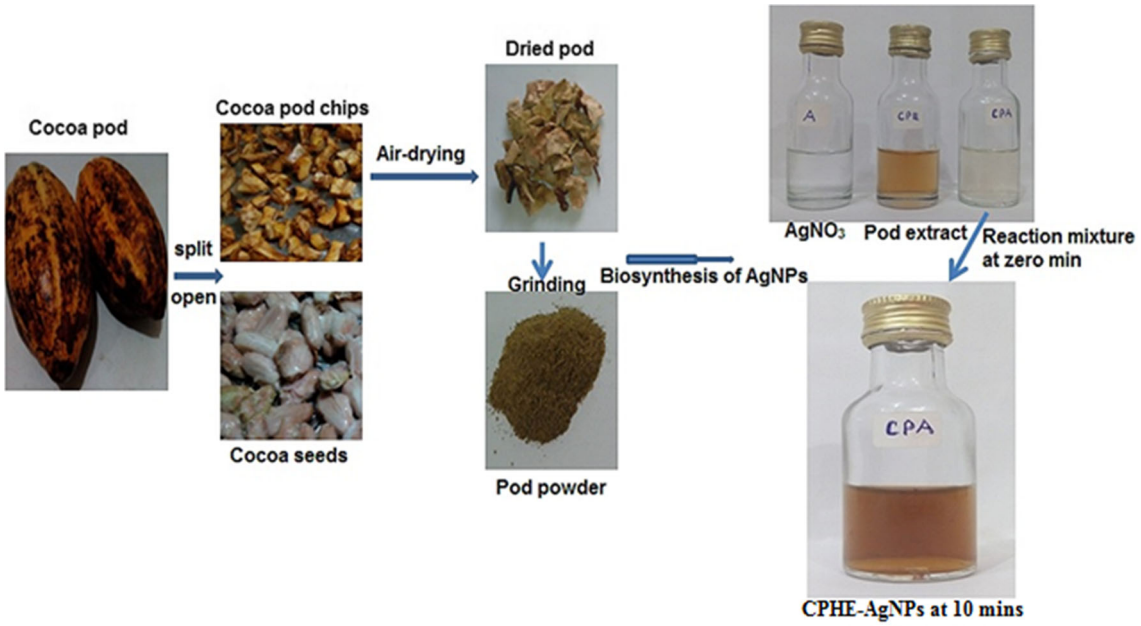

Fig. 1 Green biosynthesis of CPHE-AgNPs using the cocoa pod husk extract

using IRAffinity-1S Spectrometer to identify the functional groups of the various biomolecules that took part in the green synthesis. Transmission electron microscopy (TEM) and EDX of the synthesized CPHE-AgNPs were conducted to determine the size, morphology, nature and their elemental composition. For TEM analysis, the colloidal sample was placed on a 200 mesh hexagonal copper grid (3.05 mm) (Agar Scientific, Essex, UK) coated with 0.3 % formvar dissolved in chloroform. The grids were dried before viewing under TEM model JEM-1400 (JEOL, USA) which was operated at 200 kV to obtain the micrographs.

Antimicrobial activities of the synthesized CPHE-AgNPs

CPHE-AgNPs' antibacterial properties against clinical isolates of *Escherichia coli* and *Klebsiella pneumonia* obtained from LAUTECH Teaching Hospital, Ogbomoso were investigated using agar diffusion method as previously described [5, 10, 23, 24]. The culture broth was obtained through overnight growth in peptone water and this was used to seed the freshly prepared plates of Mueller–Hinton Agar (Lab M Ltd., UK). Thereafter, plates were bored with the aid of cork borer, and 100 µl of the graded concentrations of CPHE-AgNPs was introduced into the wells. This was subsequently followed by incubation at 37 °C for 24 h, after which zones of inhibition were measured.

Antibacterial susceptibility test

The drug susceptibility of test isolates was carried out as previously demonstrated [35, 36]. The isolates were tested on the discs (Abtek Biologicals Ltd., UK) impregnated with antibiotics containing (µg): ceftazidime (Caz), 30; cefuroxime (Crx), 30; gentamicin (Gen), 10; ceftriaxone (Ctr), 30; ofloxacin (Ofl), 5; augmentin (Aug), 30; erythromycin (Ery), 30; and cloxacillin (Cxc), 5 for Gram-positive isolates. The Gram-negative isolates were tested against antibiotics (µg): ceftazidime (Caz), 30; cefuroxime (Crx), 30; gentamicin (Gen), 10; ampicillin (Amp), 10; ofloxacin (Ofl), 5; augmentin (Aug), 30; nitrofurantoin (Nit), 300; and ciprofloxacin (Cpr), 5. After incubation at 37 °C for 48 h, the zones of inhibition were measured and interpreted [36] taking into cognizance of the recommended breakpoints [37].

Antimicrobial properties of synthesized CPHE-AgNPs as additive in paint

The antimicrobial usefulness of CPHE-AgNPs as additive in paint was investigated as described earlier [5, 10, 23], by inoculating 19 ml of sterilized commercially procured white emulsion paint with 1 ml ($\sim 10^6$ cfu/ml) of 18-h broth cultures of *E. coli, K. pneumoniae, S. pyogenes, Staphylococcus aureus* and *Pseudomonas aeruginosa*. In the case of antifungal assay, 48-h broth cultures of *A. flavus, A. fumigatus* and *A. niger* were used as inocula.

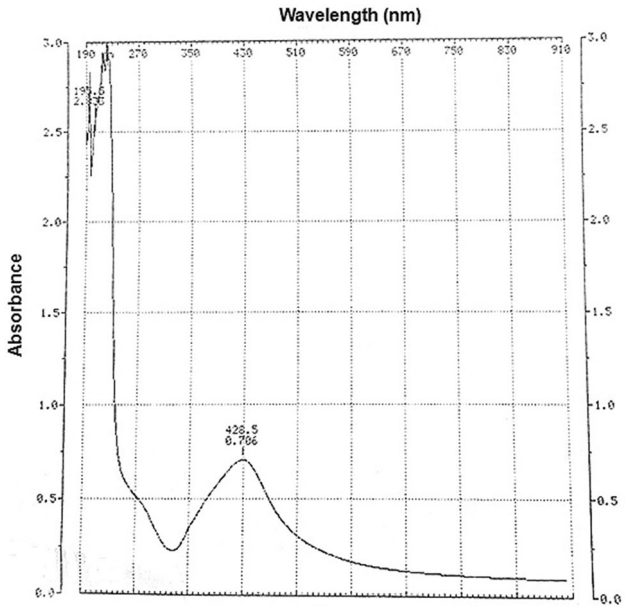

Fig. 2 The UV–vis absorption spectrum of the biosynthesized CPHE-AgNPs

The test experiment consisted of the paint and test organism, which was supplemented with 1 ml of 100 µg/ml of CPHE-AgNPs. These were incubated at 37 and 30 ± 2 °C for 48 h for bacteria and fungi, respectively. Thereafter, 1 ml of the contents of each bottle was inoculated on nutrient agar for bacteria and potato dextrose agar for fungi, and incubated appropriately for 48 h before examination for growth.

Antioxidant activities of CPHE-AgNPs

DPPH radical scavenging activity

This was carried out using the methods of William et al. [38] by reacting one milliliter of graded concentrations of CPHE-AgNPs prepared in methanol with 4.0 ml methanolic solution of 0.1 mM DPPH. The mixture was shaken and left in a dark box to stand for 30 min at room temperature (30 ± 2 °C). One milliliter of absolute methanol mixed with 4.0 ml of 0.1 mM methanolic DPPH was also prepared and used as blank. The absorbance of the resulting solution was measured at 517 nm on a UV/Vis spectrophotometer (model 6405, Jenway Ltd. Essex, UK). The inhibitory percentage of DPPH was determined accordingly [39].

$$\%\,\text{inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

The efficient concentration of CPHE-AgNPs that decreased the initial concentration of DPPH radical by 50 % (IC_{50}) was obtained by interpolation from linear regression analysis [34]. This same procedure was used for standard antioxidant compounds such as quercetin and β-carotene.

Ferric reducing activity

This was investigated using the methods of Tan et al. [40], which involved addition of 250 µl of phosphate buffer

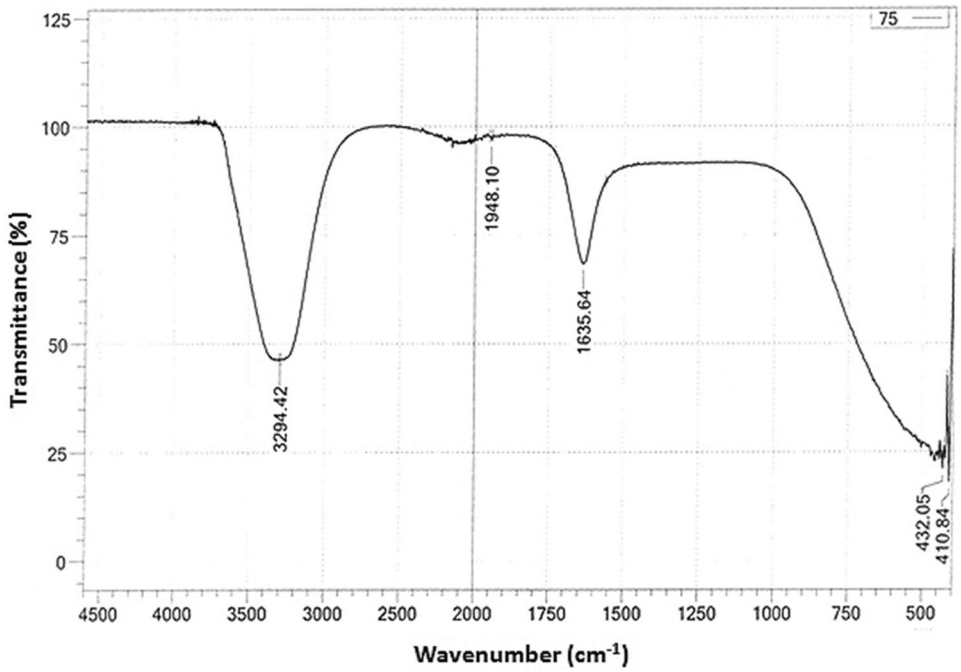

Fig. 3 The FTIR spectrum of the biosynthesized CPHE-AgNPs

(pH 6.6) and 2.5 ml of potassium ferricyanide to 1 ml of different concentrations of CPHE-AGNPs prepared using distilled water. This was followed by incubation of the resulting solution at 50 °C for 20 min. After cooling, 250 µl of trichloroacetic acid was added and centrifuged for 10 min at 500 rpm. Then, 250 µl of the supernatant with 250 µl of deionized distilled water and 500 µl of ferric (II) chloride were mixed thoroughly and absorbance was read at 700 nm. The blank was prepared with all reagents without the CPHE-AgNPs.

Larvicidal activity

This was evaluated in a dose–response bioassay against the first instar anopheline larvae as previously described [10], by exposing ten *Anopheles* mosquito larvae to 10 ml of graded concentrations of CPHE-AgNPs (10–100 µg/ml) in triplicate at room temperature (30 ± 2 °C).The number of dead larvae was recorded at specific intervals after exposure until total death was obtained. In the control experiment, the larvae were exposed to sterile distilled water

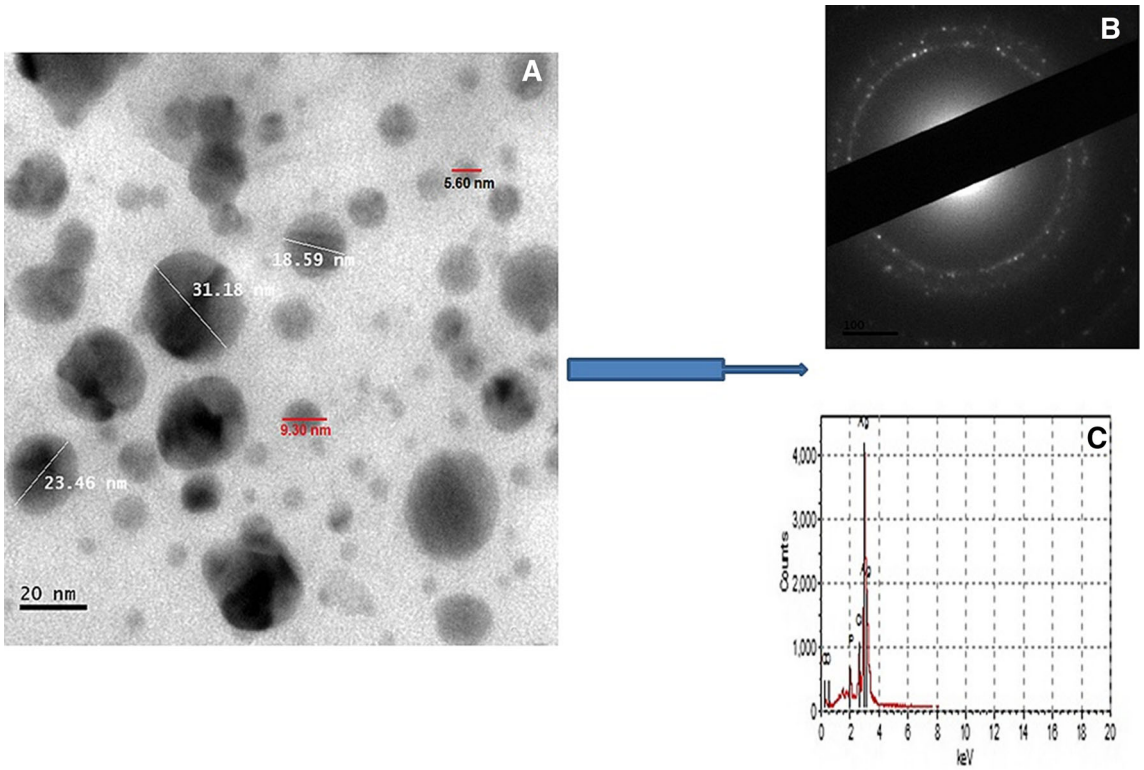

Fig. 4 Transmission electron micrograph (**a**), selected area electron diffraction pattern (**b**), and energy dispersive X-ray signal (**c**) of the biosynthesized CPHE-AgNPs

Table 1 The antibiotic resistance pattern of the test bacterial isolates

No of antibiotics	Isolates*	Source	Resistance pattern
3	KU	Urine	Crx, Gen, Amp
5	PA	Sputum	Caz, Crx, Aug, Nit, Amp
7	KW	Wound	Caz, Crx, Gen, Cpr, Ofl, Aug, Amp
8	EU	Urine	Caz, Crx, Gen, Cpr, Ofl, Aug, Nit, Amp
	SP	Sputum	Caz, Crx, Gen, Ctr, Ery, Cxc, Ofl, Aug
	SA	Ear	Caz, Crx, Gen, Ctr, Ery, Cxc, Ofl, Aug

* K, *K. pneumoniae*; PA, *P. aeruginosa*; EU, *E. coli*; SP, *S. pyogenes*; SA, *S. aureus*; antibiotics abbreviations are as defined under Experimental Details

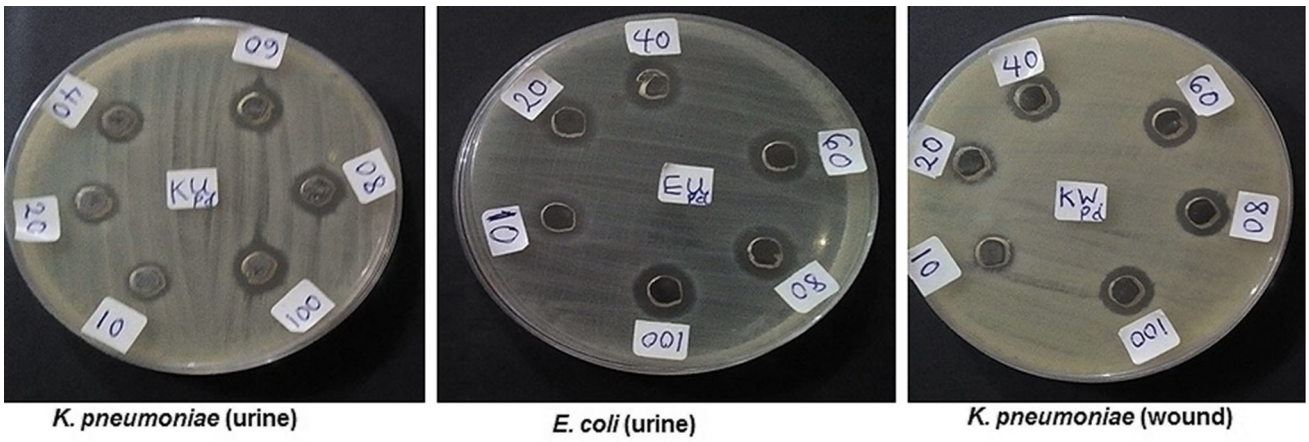

Fig. 5 The antibacterial activities of synthesized CPHE-AgNPs against some clinical bacterial isolates

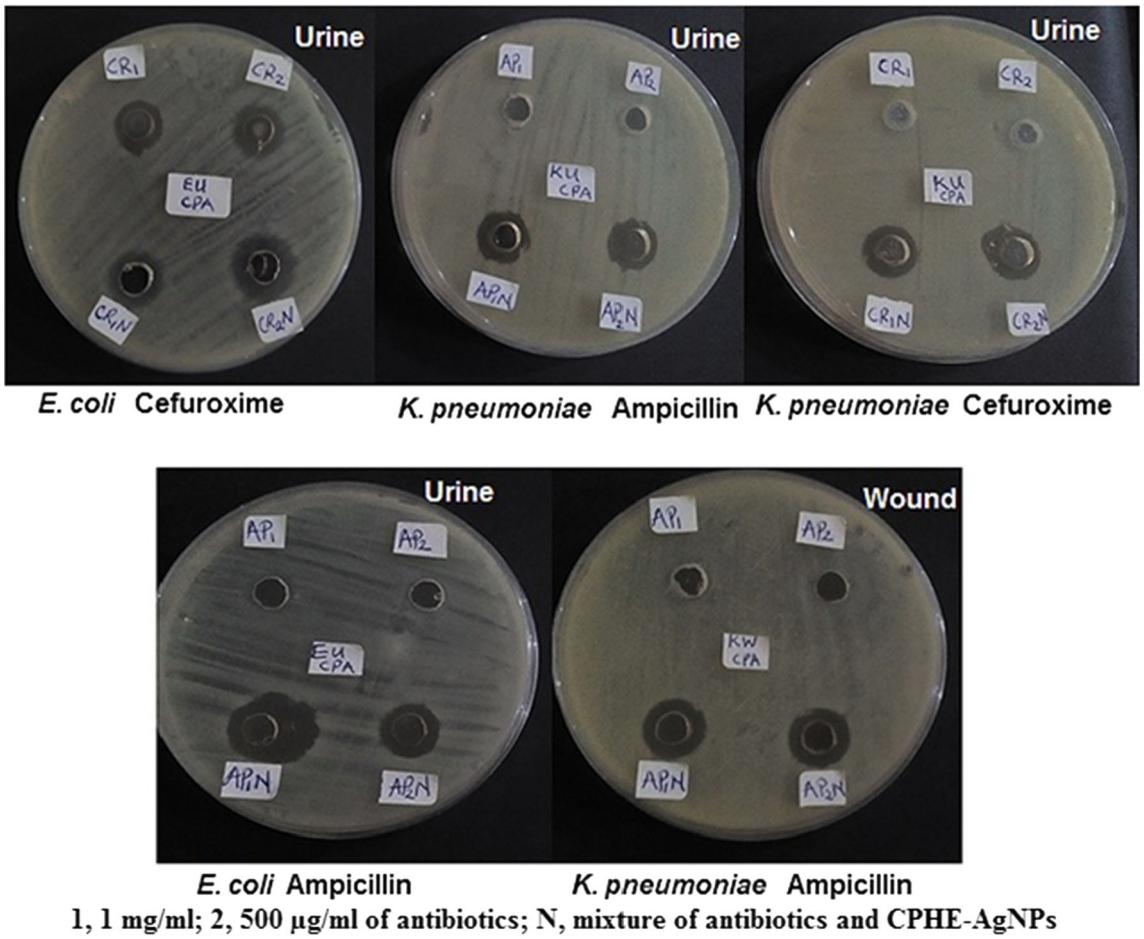

Fig. 6 The synergistic activities of synthesized CPHE-AgNPs with ampicillin and cefuroxime on some clinical bacterial isolates

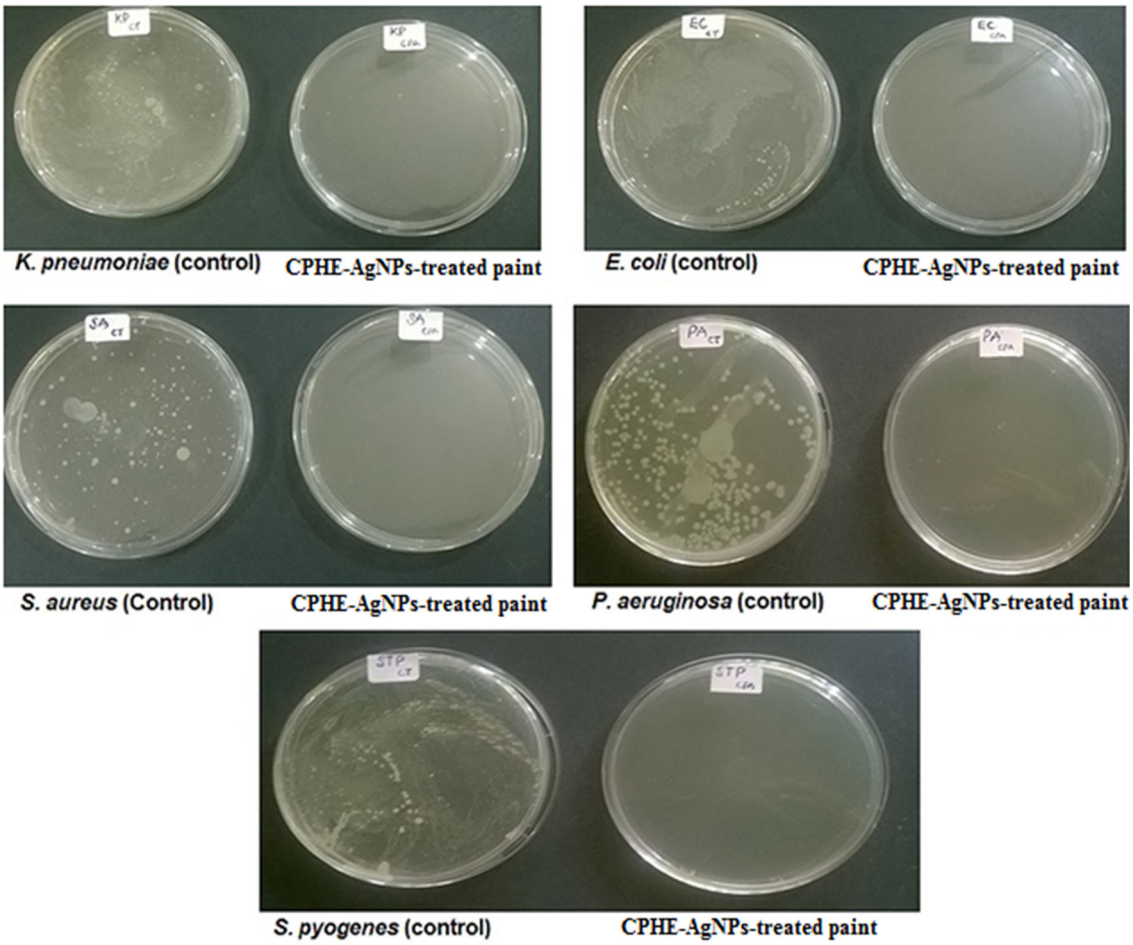

Fig. 7 Antibacterial activities of synthesized CPHE-AgNPs on bacteria inoculated into emulsion paint

under the same conditions. The percentage mortality was plotted against the concentration of the CPHE-AgNPs.

Results and discussion

Biogenic formation of CPHE-AgNPs

In the present study, CPHE mediated the formation of CPHE-AgNPs within a period of 10 min, producing brown color (Fig. 1). Colloidal green-synthesized AgNP solutions exhibiting shades of color from yellowish through brown to dark brown have been reported by several authors [5, 9, 10, 23, 24, 41, 42], suggesting the presence of different macromolecules in the extracts that played catalytic and stabilization roles in the formation of the particles. The maximum absorbance readings for the biosynthesized CPHE-AgNPs occurred at wavelength of 428.5 nm (Fig. 2). The value obtained is within the range reported for AgNPs [10, 23, 43–46]. The CPHE-AgNPs exhibited a high level of stability, devoid of aggregation or deterioration.

FTIR spectrum for CPHE mediated CPHE-AgNPs (Fig. 3) manifested strong peaks at 3294.42, and 1635.64 cm^{-1}, implicating proteins and phenolic compounds as the capping and stabilization molecules involved in the biotransformation process that produced the CPHE-AgNPs. The band 3294 is typical of N–H bond of amines, while that of 1635 is indicative of C=C stretch of alkenes or C=O stretch of amides [47]. In cocoa, total phenolic (determined at 45.6–46.4 mg gallic acid equivalent of soluble phenolic), 32.3 % carbohydrate, 21.44 % lignin, 19.2 % sugars, 8.6 % protein and 27.7 % minerals were previously reported [27], and they are biomolecules known to be very rich in the identified chemical bonds. Specifically, compounds such as citric acid, malic acid protocatechuic acid, p-hydroxybenzoic acid, salicyclic acid, kaempferol, linarin, resveratrol, apigenin, luteoin, crysoplenol, linoleic acid and oleic acid have been identified in cocoa pod extract [48].

The CPHE-AgNPs were fairly spherical in shape with sizes ranging from 4 to 32 nm (Fig. 4a), which is in agreement with those earlier reported [5, 9, 10, 23, 24, 44, 45]. The particles were well dispersed within the organic

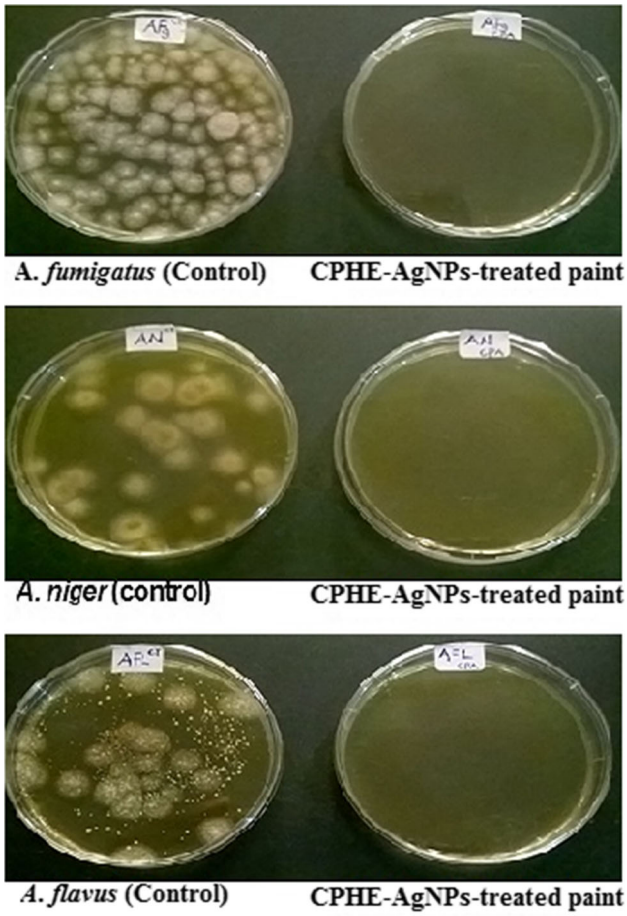

Fig. 8 Antifungal activities of synthesized CPHE-AgNPs on fungi inoculated into emulsion paint

matrix, indicating good stability against aggregation. The EDX patterns (Figs. 4c) showed the intense presence of silver in the CPHE-AgNPs colloidal solution [5, 10, 23, 24, 49, 50] to the tune of 95 %, having the ring-like SAED pattern (Fig. 4B) associated with the face-centered cubic crystalline structure of silver [47]. It can, therefore, be inferred from these results that cocoa pod husk extract is a veritable source of biomolecules in the biogenic and eco-friendly synthesis of CPHE-AgNPs that could be of wide application in the expanding field of nanobiotechnology. This report adds to bioresource utilization of agro-wastes in the synthesis of nanoparticles.

Antibacterial activities of CPHE-AgNPs against multidrug-resistant bacteria isolates

CPHE-AgNPs strongly inhibited the growth of multidrug-resistant *K. pneumoniae* and *E. coli* (Table 1) with the zones of inhibition of 10–14 mm (Fig. 5) at concentrations of 40–100 μg/ml. The activities shown by the particles against these resistant isolates are of considerable importance, indicating that the particles can be deployed to reduce the growth of drug-resistant isolates that abound in the environment [35, 36, 51–57]. Similar results on the tremendous antibacterial activities of green synthesized AgNPs have been reported [5, 9, 10, 23, 24, 47, 49].

Furthermore, the CPHE-AgNPs contributed to improvement (42.9–100 %) in the antibacterial activities of cefuroxime and ampicillin through synergy (Fig. 6). It is interesting to note that in several cases where the resistant isolates were not inhibited by the antibiotics, the CPHE-AgNPs-antibiotic treatments produced outstanding growth inhibitions against strains of *K. pneumoniae* and *E. coli.* These results concurred with those reported in similar studies [5, 10]. This pronounced activity is a further testimony to the potentials of CPHE-AgNPs in combating multidrug-resistant isolates, which would be of immense application in biomedical industry.

Antimicrobial activities of CPHE-AgNPs in paint

The incorporation of CPHE-AgNPs into emulsion paint led to effective inhibition of the growth of *E. coli, K. pneumoniae, S. pyogenes, S. aureus P. aeruginosa* (Fig. 7), *A. flavus, A. fumigatus* and *A. niger* (Fig. 8) as against the abundant growth on the control plates. We have previously shown that biosynthesized AgNPs can protect paint from microbial deterioration through antimicrobial activities [5, 10, 23], thus reiterating the relevance of AgNPs as antimicrobial additives in paint for applications in the built environment [58].

Antioxidant activities of CPHE-AgNPs

The DPPH-free radical scavenging activities of the biosynthesized CPHE-AgNPs were in the range of

Table 2 The ferric ion reducing activity of the biosynthesized CPHE-AgNPs

Test material (μg/ml)*	Ferric ion reducing power activity (%)				
	20	40	60	80	100
CPHE-AgNPs	0.1614.44	0.3733.39	0.4641.52	0.9182.13	0.9383.94
Standards (μg/ml)*	200	400	600	800	1000
Quercetin	12.050.13	20.390.22	28.730.31	63.950.69	1001.12
β-carotene	11.530.03	26.920.07	19.230.05	42.310.11	65.380.17

* Concentration; each value is an average of three readings

32.62–84.50 % at the investigated concentration of 20–100 μg/ml, while those of β-carotene and quercetin were in the range of 11.11–66.67, and 43.87–74.62 % inhibitions, respectively, at the concentrations of 0.2–1 mg/ml. The IC_{50} obtained were 49.70, 430 and 710 μg/ml for CPHE-AgNPs, quercetin and β-carotene, respectively. In the same vein, the ferric ion reducing activities of the CPHE-AgNPs were in the range of 14.44–83.94 % for concentrations of 20–100 μg/ml (Table 2), whereas β-carotene had activities in the range of 11.53–65.38 %, and quercetin displayed ferric ion reduction in the range of 12.05–100 % at concentrations of 0.2–1.0 mg/ml. These antioxidant activities shown by the CPHE-AgNPs are similar to those previously reported [10, 23, 59, 60], with the particles manifesting greater potencies than the antioxidant standards. Previous studies have established the antioxidant capabilities of extracts obtained from cocoa pod husk [28, 34, 48]. Generally, the free radical scavenging activities of AgNPs have been attributed to the bioreductant molecules present on the surface of the nanoparticles which increase the surface areas for antioxidant activity [60]. The presence of compounds such as citric acid, malic acid, terpenoid and resveratrol has been previously attributed to antioxidant activities of cocoa pod extracts [48].

Larvicidal activity of CPHE-AgNPs

CPHE-AgNPs showed potent larvicidal activities (70–100 %) against the larvae of *Anopheles* mosquito at concentrations of 10–100 μg/ml within 2 h (Fig. 9) with the LC_{50} of 43.52 μg/ml. The larvicidal activity of CPHE-AgNPs is similar to those previously reported on the larvicidal activities of some plant and bacterial extract-mediated AgNPs on *Anopheles* larvae [10, 22, 61, 62]. However, in the present study, the potency of the CPHE-AgNPs was found to be greater within 2 h of exposure than those previously reported. It can, therefore, be inferred from these results that the biosynthesized CPHE-AgNPs can find useful application in the malaria control programme by killing the larvae of the vector of *Plasmodium* parasites.

Conclusion

The present study has clearly demonstrated the usefulness of cocoa pod husk extract (CPHE) as a cost-effective and eco-friendly bio-resource in the green synthesis of CPHE-AgNPs. The synthesized particles were fairly spherical with size ranging from 4 to 32 nm. The highly remarkable antibacterial activities of CPHE-AgNPs and enhanced activities in synergy with antibiotics against multidrug-resistant clinical isolates of bacteria, including excellent larvicidal activities against larvae of the vector of *Plasmodium* parasites indicated the possibility of their exploitation in biomedical industry. Furthermore, the successful inhibition of microbial growth when used as additive in paint and very strong antioxidant activities suggests the biotechnological potential of the synthesized nanoparticles in the biomedical industry and as a coating for surfaces of materials to protect them against microbial growth while improving their shelf life. To the best of our knowledge, this report represents the first reference to cocoa pod husk extract in the green synthesis of CPHE-AgNPs.

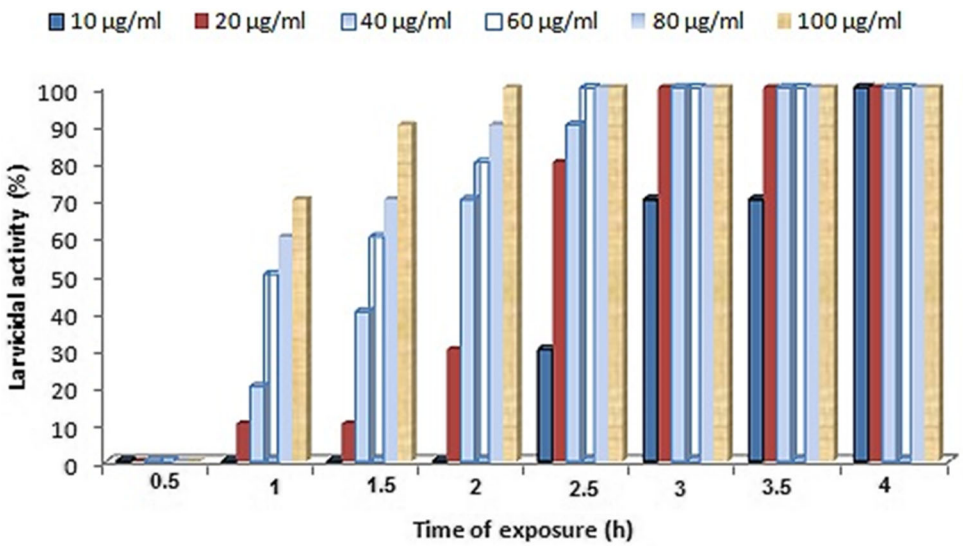

Fig. 9 Larvicidal activity of the biosynthesized CPHE-AgNPs on *Anopheles* mosquito larvae

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