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Research article

Biosynthesis of copper nanoparticles from areca nut extract and its antibacterial and antioxidant properties

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Abstract

The phytochemicals present in different maturity stages of areca nut (*Areca catechu*) were evaluated. Phytochemical tests revealed the presence of carbohydrates, alkaloids, flavonoids, tannins and phenolic compounds. Copper nanoparticles were biosynthesized from the matured areca nut extract and characterization of the synthesized nanoparticles was carried out by analyzing the surface plasmon resonance, particle size and zeta potential. Field emission scanning electron microscopy and Energy dispersive X-ray analysis (EDAX) of nanoparticles synthesized from only stage 5 areca nut extract were used because this stage produced the highest antibacterial activity. The antibacterial activity of nanoparticles and the extract was tested against Gram-positive and Gram-negative bacteria; the plain extract had a zone of inhibition of 10–13 mm, whereas biosynthesized copper nanoparticles had a zone of inhibition of 23–25 mm. The nanoparticles also had enhanced antioxidant activity compared to plain areca nut extract and thus, the extract has potential for use as an antioxidant agent in pharmaceuticals and cosmetics. It can be concluded from this study that by using a nanotechnological approach, phytochemicals found in areca nut could be put to good use to enhance their antibacterial and antioxidant activity.

Introduction

Areca nut (*Areca catechu*) is a plant of tropical and subtropical countries having major economic importance as a commercial cash crop (Naagarajan and Meenakshi, 2016). Areca nut is used in combination with beetle nut for chewing and as a mouth freshener (Ling et al., 2001). Areca nut has been used in various kinds of traditional medications for the treatment of diseases such as, schizophrenia and glaucoma and it is also known to be a stimulant and aids in digestion (Rama et al., 2016). Chewing betel quid improves oral hygiene, increases salivary secretion and the motility of food and reduces absorption of food thereby preventing weight gain (Chempakam 1993; Park et al., 2002). It is also said to be anti-diabetic and to reduce the cholesterol level (Anthikat and Michael, 2009). Areca nut extract contains substantial amounts of phytochemicals such as carbohydrates, flavonoids, tannins, alkaloids, polyphenols, which can be harnessed to develop pharmaceutical products (Bhat et al., 2015). When phytochemicals bind to the surface of nanoparticles they act as free-radical scavengers, nonspecific reducing agents and a source for ligand binding (Das et al., 2016). In this respect, flavonoids and

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phenols have the inherent ability of scavenging free radicals, which is principally based on the redox properties of their phenolic hydroxyl groups (Barros et al., 2007). The search for natural antioxidants of plant origin has increased remarkably for application in pharmaceuticals (Skerget et al., 2005). Nanotechnology is one of the newest and most promising areas of research in modern medical science, which deals with nanoparticles applications in medical devices and therapy because nanoparticles exhibit improved properties based on their size, distribution and morphology (Dipankar and Murugan, 2012). There is increasing demand for green-synthesized nanoparticles to be used for medical applications (Lukman et al., 2011). Nanoparticles synthesized biologically are known to be advantageous over chemical and physical methods of synthesis as they are environment-friendly because the need to use high-pressure, high temperature and toxic chemicals is nullified (Jain et al., 2009). Good manipulation and control over the growth of nanoparticles can be achieved using plant extracts (Mittal et al., 2014). Recent investigations include green synthesis of nanoparticles using honey (Balasooriya et al., 2017) and *Allophylus serratus* leaf and leaf-derived callus extract for synthesis of nanoparticles (Jemal et al., 2017). The extracellular synthesis of copper nanoparticles using *Streptomyces species* and its application as an antimicrobial agent has been investigated but culture maintenance is a laborious task and scaling up is challenging (Usha et al., 2010). Theivasanthi and Alangar (2011) carried out the synthesis of copper nanoparticles using electrolysis and studied their application as an antimicrobial agent using a direct electric current to drive the non-spontaneous chemical reaction, thereby increasing the energy requirements for synthesis of nanoparticles. Water is a suitable solvent for the extraction of phytochemicals present in areca nut seed when compared to organic solvents like methanol used for the synthesis of silver nanoparticles (Bhat et al., 2015). Consequently, water was used in the current study as a viable and economical option for large scale production of stable nanoparticles. Copper sulphate mixed with lime is sprayed on areca nut as Bordeaux mixture to control *Phytopthora* infestation (Matthew et al., 2015) and this could lead to the absorption of copper ions into the areca nut during growth, remain there subsequently. On this premise, it was thought that copper ions could be responsible for antimicrobial activity and it is well documented that copper acts as an antimicrobial agent (Leite and Padoveze, 2012; Hobman and Crossman, 2014). Hence, the present study was carried out on biosynthesis of Cu nanoparticles using the aqueous extract of areca nut to study antibacterial and antioxidant properties. These nanoparticles have the capacity to inhibit the growth of both Gram-positive and Gram-negative bacteria which can be explored further for use in cosmetics. Increased antioxidant capacity of these biosynthesized nanoparticles increases their potential to be used in preparations of cosmetics and pharmaceutical products.

Materials and Methods

Preparation of extract

Different maturity stages (Fig. 1) of areca nuts were collected from a farm in Udupi district, Karnataka state, India, and the outer fibrous cover was peeled. The fresh nuts were crushed into small pieces using a mortar and pestle. A solution of 10% (weight per volume; w/v) concentration was prepared by boiling the crushed pieces of areca nut (100°C, for 30 min) from each maturity stage (stages 1, 2, 3, 5) in distilled water. Each solution was filtered using Whatman filter paper-1 and the filtrate was used for the analysis (Fig. 2). Extract obtained when moderately matured areca nuts are boiled in water as a process for its consumption with beetle leaves was directly collected from the areca nut processing center, Shirsi, North Karnataka, Karnataka state, India, and considered as Stage 4.

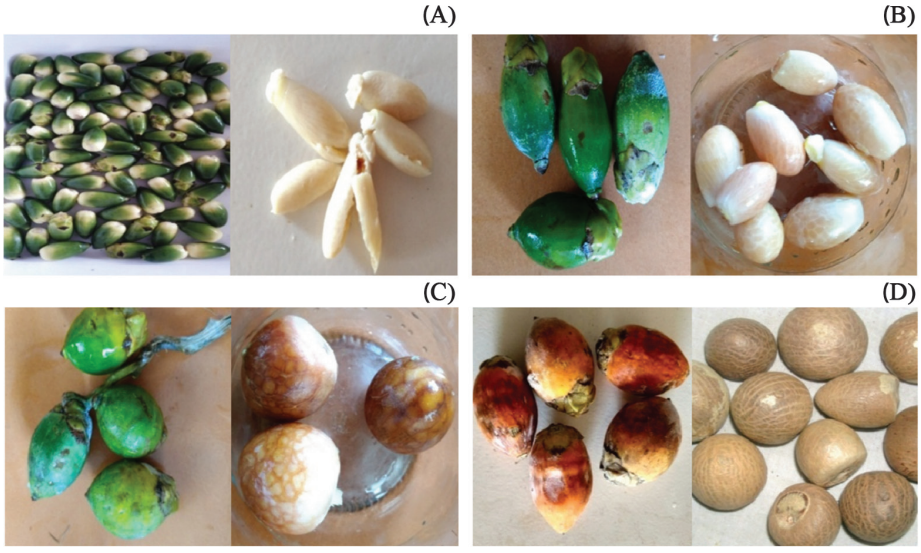

Fig. 1 Different stages of maturation of areca nut: (A) tender areca nut (stage 1); (B) slightly mature areca nut (stage 2); (C) partially mature areca nut (stage 3); (D) mature areca nut (stage 5)

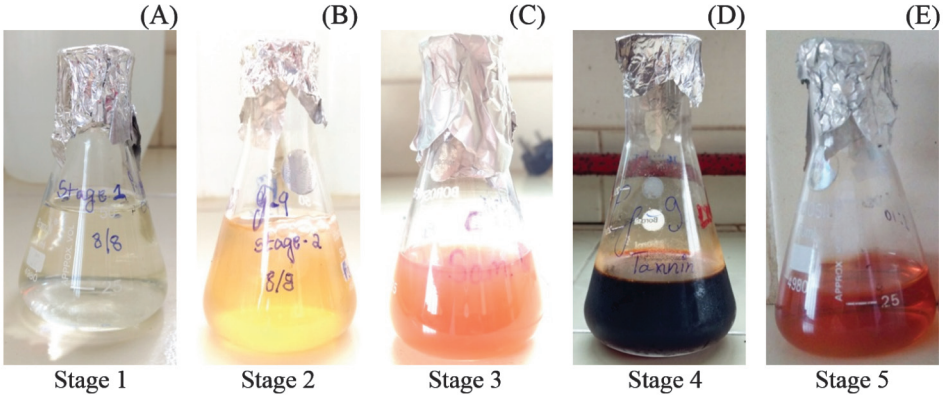

Fig. 2 Extracts of different maturity stages of areca nut

Phytochemical screening

The method of Kokate et al. (2008) was used. Chemicals used in the present study were of analytical grade and were obtained from E. Merck, India.

Carbohydrate testing

Molisch's test

A sample (2 mL) of areca nut extract was taken in a test tube and 1 mL of freshly prepared 5% alcoholic solution of α- naphthol was added followed by 1 mL of concentrated sulfuric acid from the side of the test tube in order to form a layer below the mixture. The appearance of a purple ring at the junction indicates the presence of carbohydrates, which disappears on the addition of excess alkali (Foulger, 1931).

Anthrone test

A sample (2 mL) of areca nut extract was taken in a test tube to which was added 2 mL of anthrone reagent (200 mg of anthrone dissolved in 100 mL of ice cold 95% H_2SO_4). The formation of a green or blue colored solution indicates the presence of carbohydrates (Dreywood, 1954).

Protein testing

Ninhydrin test

A sample (1 mL) of the extract was taken in a test tube to which 1mL of ninhydrin solution was added and then kept in a boiling water bath for 5 min. The formation of a purple or pink color indicates the presence of amino acids and the formation of yellow color indicates the presence of amino acids such as proline (Friedman, 2004).

Bradford's test

A sample (1 mL) of the extract was taken in a test tube to which was added 2–3 drops of Bradford's reagent). Appearance of blue color indicates the presence of proteins (Bradford, 1976).

Alkaloid testing

Hager's test

A sample (1 mL) of the extract was taken in a test tube and few drops of Hager's reagent (saturated solution of picric acid) was added. Formation of yellow precipitate confirms the presence of alkaloids (Nilanjana et al., 2013).

Wagner's test

A few drops of Wagner's reagent (solution of iodine in potassium iodide) were added to 1 mL of the extract. A yellow or brown precipitate indicates the presence of alkaloids (Nilanjana et al., 2013).

Flavonoid testing

Ferric chloride test

The test solution (extract) was treated with a few drops of ferric chloride. Formation of a blackish red precipitate indicates the presence of flavonoids (Nilanjana et al., 2013).

Lead acetate solution test

A few drops of 10% lead acetate solution were added to the test solution (extract). The formation of a yellow color indicates the presence of flavonoids (Deb et al., 2013).

Tannin testing

A few drops of 5% (w/v) $FeCl_3$ solution was added to 2mL of the extract. The appearance of green color indicates the presence of tannins (Deb et al., 2013).

Biosynthesis of copper nanoparticles

A sample (0.004 g) of copper sulfate was weighed and added to 25 mL of 1:10 diluted 10% extract of areca nut at different maturity stages such that the concentration of copper sulfate in the solutions was 1 mM. These mixtures were kept on a shaker maintained at 100

revolutions per minute (rpm) at room temperature and absorbance was recorded hourly. The color change in the solution was measured at regular intervals based on measuring the surface plasmon resonance from 400–700 nm using an ultraviolet-visible spectrophotometer. The solution was centrifuged at 10,000 rpm at 4°C for 20 mins, and the obtained pellets were washed twice with distilled water and once with ethanol. Purified pellets (copper nanoparticles) were dried by keeping them in a desiccator overnight.

Characterization of nanoparticles

Ultraviolet-visible spectroscopic analysis

Ultraviolet-visible spectroscopy was used to carry out surface plasmon resonance of the synthesized copper nanoparticles.

Particle size and zeta potential

A Zetasizer Nano Instrument (Malvern) was used for data acquisition for the estimation of the particle size distribution as well as to determine the zeta potential distribution of the colloidal solution of the copper nanoparticles.

Field emission scanning electron microscopy and Energy dispersive X-ray analysis (EDAX)

Size and morphology of copper nanoparticles were examined using a field emission scanning electron microscope (Carl Zeiss) by sputtering. The microstructure of the sputtered samples was analyzed at 5 KV. Specimen preparation was done by taking a small amount of nanoparticles powder dispersed on carbon tape and passing a 20 mA current for 10 min to obtain a thin gold coating over the sample. Energy dispersive X-ray analysis (EDAX) was used for elementary composition analysis.

Antibacterial activity

The antibacterial activity of biosynthesized copper nanoparticles was tested on Gram-positive (*Bacillus subtilis*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria. For antimicrobial assay, fresh microbial colonies (100 µL) were inoculated into 10 mL of nutrient broth medium. After 16–18 hr of incubation the number of colony forming units per milliliter (cfu/mL) was calculated for $1{:}10^5$ dilutions which corresponded to 9.1×10^8 cfu/mL and 6.6×10^8 cfu/mL for *B. subtilis* and *Ps. aeruginosa*, respectively. Bacterial nutrient agar plates were prepared; the spread plate culture of each bacterium was prepared by taking 50 µL of fresh culture of the bacterium. Using an agar punch, a 5 mm well was punched at the center of each Petri dish. Copper nanoparticles at a concentration of 10 mg/mL were suspended in dimethyl sulfoxide (DMSO) and 50 µL of the suspension was loaded into the wells to study the antibacterial activity using the agar diffusion method. A sample of 50 µL of DMSO, areca nut extract and 1 mM aqueous solution of $CuSO_4$ were also loaded in wells as a control. A standard antibacterial drug (Ciplox; Cipla India Ltd.) was used as a control. Light zones around each well were considered as the zone of inhibition, which was measured in millimeters from the periphery of the well to the periphery of the zone.

Antioxidant activity of copper nanoparticles

Determination of total phenolic acid

The total phenolic acid content of samples of different maturity stages of areca nut and nanoparticle solution was measured using the methods of Wang et al. (1997) and Zhishen et al. (1999). One mL extract and nanoparticle solution was mixed with 2 mL of 1:10 diluted Folin-Ciocalteu reagent, followed by 2 mL of 7.5% sodium carbonate. The reaction mixture left to stand at room temperature for 90 min. The greenish-blue complex that developed was measured spectrophotometrically at 750 nm against the blank. The total phenol content of the extract and nanoparticle solution was expressed in terms of equivalents of gallic acid in micrograms per milligram of dry mass (EGA µg/mg dry mass).

Determination of total flavonoid content

The method of Prieto et al. (1999) was used. Five mL extract and nanoparticle solution was mixed with 300 µL of 5% sodium nitrite and 300 µL of 10% aluminum chloride, followed by the addition of 2 mL of 1 M sodium hydroxide. The reaction mixture was then incubated at room temperature for 6 min. Absorbance was measured at 510 nm against the blank. The total flavonoid content of the sample was expressed in terms of equivalents of quercetin in micrograms per milligram of dry mass (QE µg/mg dry mass).

Total antioxidant capacity

The method of Braca et al. (2001) was used. The total antioxidant capacity of areca nut extract and nanoparticle solution was determined using the phosphomolybdenum method. Three hundred µL was added to 3 mL of reagent mixture consisting of 4 mM ammonium molybdate, 0.6 M sulfuric acid and 2 mM of sodium phosphate. Reaction mixtures were incubated at 95°C for 90 min and after cooling, the absorbance was measured at 695 nm against a blank. The antioxidant capacity was expressed as equivalents of ascorbic acid.

2-Diphenyl-1-picrylhydrazyl radical scavenging activity

The method of Chang et al. (2002) was used. Areca nut extract and nanoparticle solution were screened for free radical scavenging activity based on the scavenging ability of 2-diphenyl-1-picrylhydrazyl (DPPH). To each test sample (extract and nanoparticle solution) 3 mL of 0.004% DPPH in 95% ethanol was added and the mixture was incubated for 30 min at room temperature in the dark. The scavenging activity of the extract and nanoparticle solution was determined by measuring the absorbance at 517 nm. The DPPH radical scavenging activity of butyl hydroxyl anisole was assayed for comparison. Radical scavenging activity was calculated using Equation 1:

$$\% \text{ Inhibition} = \{[(A_c - A_t) / A_c]\} \times 100 \quad (1)$$

where, A_c is the absorbance of the control reaction and A_t is the absorbance of the test sample.

Hydrogen peroxide scavenging activity

The method of Ruch et al. (1989) was used. A solution of 100µM hydrogen peroxide was prepared in phosphate buffered saline (pH 7.4). One mL and standard ascorbic acid were added to 1.2 mL of 100 µM H_2O_2. After incubating for 10 min, the absorbance was measured at 230 nm. The percentage of inhibition was calculated using Equation 1:

$$\% \text{ Inhibition} = \{[(A_c - A_t) / A_c]\} \times 100 \quad (1)$$

where, A_c is the absorbance of the control reaction and A_t is the absorbance of the test solution reaction.

Results and Discussion

Phytochemical screening

The phytochemical tests conducted for the areca nut extract showed the presence of carbohydrates, alkaloids, tannins and flavonoids. These phytochemicals are responsible for antimicrobial properties and act as antioxidants thereby facilitating inhibition of free radicals (Das et al., 2016).

Characterization of biosynthesized copper nanoparticles

Characterization using ultraviolet-visible spectrophotometry

Change in the color of the solution from orange to brown indicated the formation of copper nanoparticles (Fig. 3A). The spectrophotometric readings at 450 nm confirmed the production of copper nanoparticles in the areca nut extracts of stages 3, 4 and 5 (Fig. 3B), however, nanoparticles synthesized using mature (stage 5) areca nut extract had better results compared to the other stages. A peak was observed in the fifth hour, after which the absorbance decreased. It was confirmed that synthesis of nanoparticles was completed by the fifth hour.

Particle size distribution and zeta potential

If nanoparticles have a smaller particle size diameter, then there is better antibacterial activity and if the zeta potential has a greater negative value, then the nanoparticles are more stable (Kim et al., 2007). When the nanoparticles synthesized from three different maturity stage extracts of areca nut were compared, the nanoparticles synthesized from the third stage had a smaller particle size compared to the other two stages, whereas the zeta potential of the third stage had a lower negative value compared to the other two stages (Table 1).

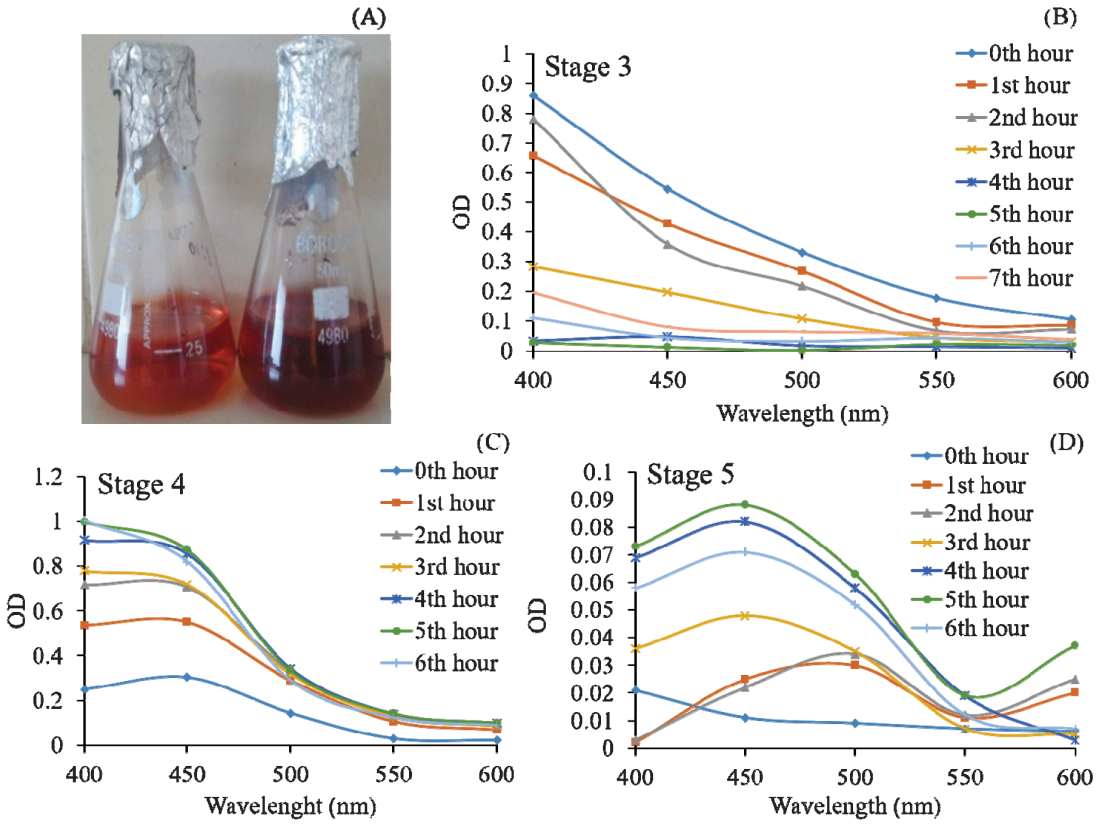

Fig. 3 (A) Change in extract color indicating the presence of copper nanoparticles. Ultraviolet-visible spectra of copper nanoparticles synthesized using: (B) stage 3 areca nut extract; (C) stage 4 areca nut extract; (D) stage 5 areca nut extract, where OD = optical density

Table 1 Particle size distribution and zeta potential values

Stage	Particle size diameter (nm)	Zeta potential (mV)
Stage 3 nanoparticles	255	-6.9
Stage 4 nanoparticles	1,203	-21.2
Stage 5 nanoparticles	337	-10.8

Thus, the third stage nanoparticles were unstable and could agglomerate. The particle size distribution of nanoparticles synthesized from Stage 4 areca nut was comparatively larger, hence the nanoparticles synthesized from matured (Stage 5) areca nut were used in the further studies (Fig. 4).

Field emission scanning electron microscopy (FESEM) and Energy dispersive X-ray analysis (EDAX)

Fig. 5A shows that the copper nanoparticles formed were spherical in shape, with a size range of 100–200 nm. For comparison, the copper nanoparticles synthesized using *Artabotrys odoratissimus* leaf extract had an average size of 100–230nm (Umesh and Gajera, 2014) indicating that the copper nanoparticles biosynthesized in the present study from areca extract were quite comparable. EDAX confirmed the purity of the sample by giving its elemental composition (Fig. 5B).

Antibacterial activity

The antibacterial activity of the extract and nanoparticles was tested against Gram-positive *(B. subtilis)* and Gram-negative (*Ps. aeruginosa*) bacteria which indicated that the antibacterial activity of the copper nanoparticles was better than that of the plain extract (Table 2 and Fig. 6).

Table 2 Zone of inhibition of areca nut extract and copper nanoparticles in mm

Sample	Gram positive	Gram negative
Dimethyl sulfoxide	-	-
$CuSO_4$	5±0.57	5±0.63
Standard antibacterial drug	48±0.67	48±0.72
Extract (stage 5)	11±0.74	13±0.69
Copper nanoparticles	25±0.59	23±0.62

Values are mean ± SD

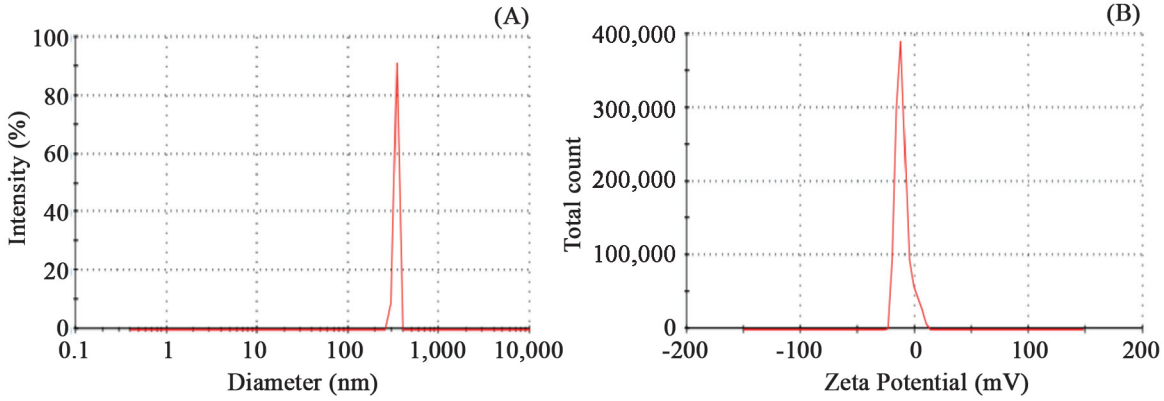

Fig. 4 Fifth stage copper nanoparticles: (A) particle size distribution; (B) zeta potential

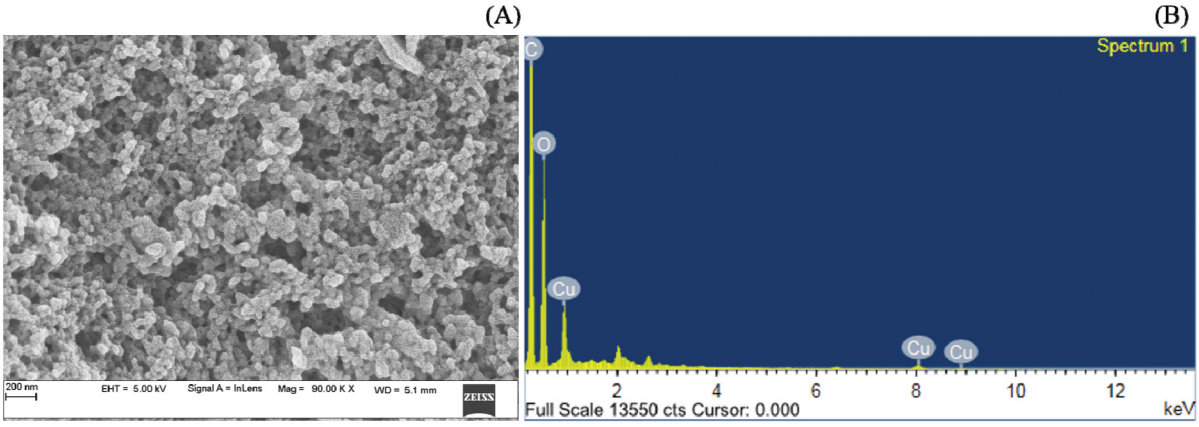

Fig. 5 Copper nanoparticles synthesized using stage 5 areca nut extract: (A) field emission scanning electron micrograph; (B) Energy dispersive X-ray analysis (EDAX) showing elemental composition

Antioxidant activity

Total phenolic acid assay

The total phenolic acid assay conducted for the different maturity stages of areca nut extract and nanoparticles synthesized revealed that the nanoparticles in the stage 5 extract had the greatest amount of phenolic acid compared with nanoparticles produced from the other stages of development of areca nut (Fig. 6A). The phenolic acid content coated over the nanoparticles was 0.019 EGA µg/mg and that of the extract was 0.0202 EGA µg/mg. The phenolic content of nanoparticles was comparable with that of the extract which indicated the capping of the nanoparticles with the polyphenols present in the extract (Barros et al., 2007).

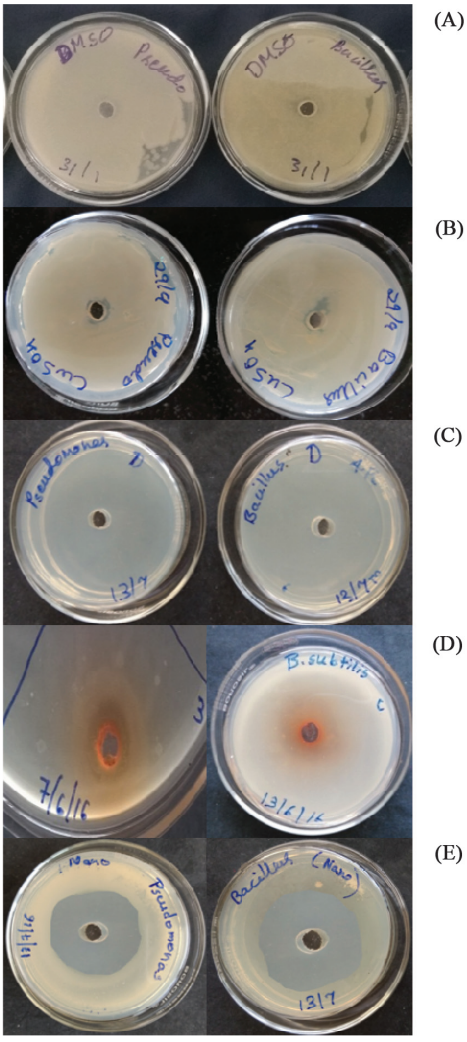

Fig. 6 Zones of inhibition on *Pseudomonas aeruginosa* (left) and *Bacillus subtilis* (right) for: (A) dimethyl sulfoxide; (B) $CuSO_4$; (C) standard antibacterial drug (50 µL of concentration at 10 mg/mL); (D) areca nut extract (50 µl of 1:10 diluted 10% extract); (E) copper nanoparticles (50 µL of concentration at 10 mg/mL)

Total flavonoid content

The assay (Fig. 7B) showed that the nanoparticles synthesized from stage 5 extract had a total flavonoid content of 3.482 QE µg/mg and that of the extract was 8.92 QE µg/mg, indicating that the nanoparticles were coated with flavonoids and hence a higher value was recorded in the assay of nanoparticles.

Total antioxidant capacity assay

The assay revealed that the nanoparticles synthesized from stage 5 of matured areca nut more total antioxidant capacity than the nanoparticles synthesized from extracts of the other stages (Fig. 7B). The total antioxidant capacity of the stage 5 nanoparticles was 31.62 EAA µg/ml and that of the extract was 26.89 EAA µg/ml indicating that the nanoparticles had better total antioxidant capacity than the extract.

2-Diphenyl-1-picrylhydrazyl radical scavenging activity

The radical scavenging activity (Fig. 7C) of stage 5 extract was 51.85%, which was greater than for the other stages of nut maturation. The nanoparticles synthesized from stage 5 extract had an absorbance value that was greater than the control samples, resulting in a negative value for the percentage inhibition (Not shown in the Fig.).

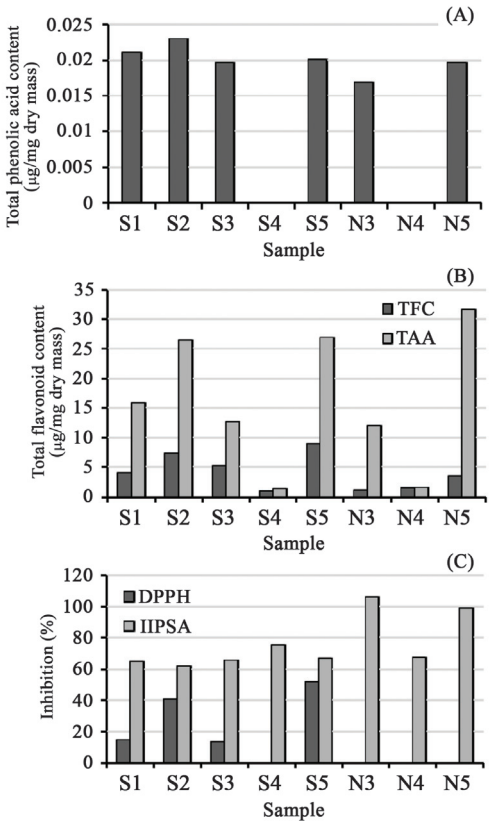

Fig. 7 Areca nut extracts and copper nanoparticles: (A) total phenolic acid (TPA) content, (B) total flavonoid content (TFC) and total antioxidant capacity (TAA); (C) 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and hydrogen peroxide scavenging activity (HPSA), where S1 = stage 1 extract; S2 = stage 2 extract; S3 = stage 3 extract; S4 = stage 4 extract; S5 = stage 5 extract; N3 = nanoparticles from third stage extract; N4 = nanoparticles from fourth stage extract; N5 = nanoparticles from fifth stage extract.

Hydrogen peroxide scavenging activity

The assay (Fig. 7C) revealed that the nanoparticles produced from the stage 3 extract had greater H_2O_2 scavenging activity than the nanoparticles synthesized from extracts of the other stages of areca nut. The H_2O_2 scavenging activity of the nanoparticles synthesized from stage 3 extract was 106.01% and that of the extract was 65.49%, indicating that the nanoparticles have higher hydrogen peroxide scavenging activity compared to the extract.

The areca nut extracts contained industrially important phytochemicals such as, alkaloids, tannins, phenols and flavonoids. The organic reducing agents present in the cells of the pure areca nut extract were capable of reducing the copper ions to neutral atoms, which then nucleated to become the nanoparticles. Among the different maturity stages of areca nut, fully matured areca nut extract produced copper nanoparticles which had antioxidant, free radical scavenging activity, indicating that nanoparticles biosynthesized from areca nut could be used as antioxidant and free radical scavenging agents in pharma products.

From the present study, it was evident that copper nanoparticles can be biosynthesized using areca nut extract and these nanoparticles are capable of inhibiting the growth of Gram-positive *(Bacillus subtilis)* and Gram-negative (*Pseudomonas aeruginosa*) bacteria compared to the unmodified extract. The nanoparticles also had antioxidant activity which could lead to their utilization in pharmaceuticals and cosmetics production. If the arecanut extract which otherwise would have gone waste if used as raw material this would provide a good example of "Waste to Wealth" through the production of nanoparticles.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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