International peer reviewed open access journal

Journal of Medical Pharmaceutical and Allied Sciences

Journal homepage: www.jmpas.com CODEN: JMPACO

Research article

Phytochemically enriched male papaya flowers: a better green candidate for silver nanoparticle synthesis and exploring its antibacterial potency

Harsha Haridas Ellathuveettil Swaminathan[1], Susmita Bhattacharya[2], Ravi Varma Mundakkara Kovilakam[1], Baiju Govindan Nair[3], Abey Joseph[3], Goutam Kumar Chandra*[1]

[1] Department of Physics, National Institute of Technology Calicut, Kozhikode, Kerala, India.
[2] Radiation Laboratory, University of Notre Dame, Notre Dame, Indiana , United states of America USA.
[3] Bio nano Engineering Laboratory, School of Biotechnology, National Institute of Technology Calicut, Kozhikode, Kerala, India.

Corresponding author: Goutam Kumar Chandra ✉ goutam@nitc.ac.in, **Orcid Id**: https://orcid.org/0000-0002-6096-7075
Department Physics, National Institute of Technology Calicut, Kozhikode, Kerala, India

Received - 23-03-2023**, Revised** - 12-05-2023, **Accepted** - 17-06-2023 **(DD-MM-YYYY)**

Refer This Article

Goutam Kumar Chandra, Harsha Haridas Ellathuveettil Swaminathan, Susmita Bhattacharya, Ravi Varma Mundakkara Kovilakam, Baiju Govindan Nair, Abey Joseph, 2023. Phytochemically enriched male papaya flowers: a better green candidate for silver nanoparticle synthesis and exploring its antibacterial potency. Journal of medical pharmaceutical and allied sciences, V 12 - I 3, Pages - 5796 – 5802. Doi: https://Doi.org/10.55522/jmpas.V12I3.5018.

ABSTRACT

Innovating green pathways for fabricating metal nanoparticles (MNPs) turns into an essential subject to promote sustainability and protect our environment from toxicity. *Carica papaya* is one of the most common cultivations with enriched phytochemicals. Here in this work, we have used male *Carica papaya* extract (CPE) from its flowers to synthesize eco-friendly silver nanoparticles (AgNPs). The physicochemical properties of CPE-reduced AgNPs (CPE-AgNPs) were studied by different spectroscopic techniques. The characteristic property of AgNPs has been confirmed by analyzing the surface plasmon resonance (SPR) band of CPE- AgNPs: at around 440 nm. The capping of as-prepared AgNPs mediated by CPE was evaluated based on the vibrational bands of functional groups by Fourier Transform Infra-Red (FTIR) spectrometer. The elemental silver composition present in CPE-AgNPs was evidently seen from the corresponding Energy Dispersive X-ray spectroscopic (EDS) measurements. Studies obtained from Transmission Electron Microscopy (TEM) images of CPE-AgNPs reveals that the majority of the CPE-AgNPs are spheres of 11.10 ± 2.30 nm diameter. The Zeta potential value for CPE-AgNPs prepared using 2 ml of CPE was 65.0 ± 2.1 mV, which ensured better stability, and the sample was selected for further studies. CPE-AgNPs show better antibacterial efficacy against bacterial strains and suggest that they can be used for developing AgNPs based medical devices such as urinary catheters to treat urinary tract infections (UTIs).

Keywords: Antibacterial, Carica papaya male flower, Green synthesis, Silver nanoparticles.

INTRODUCTION

Green nanotechnology is an unavoidable linkage between nanotechnology and nature [1]. The reduction, stabilization and functionalization of nanomaterials using different parts of plants become more relevant due to minimizing environmental contamination created by the usage of toxic chemicals in other methods [2]. Green reduced AgNPs are becoming one of the attentive areas of the bio-nanotechnology branch nowadays because of the remarkable innovations and applications extended from the growth restriction of microorganisms to the furtherance of electronic circuits [3].

The urge to develop renewable materials and environmentally benign solvents adds the need for green synthesized AgNPs to apply in various fields, such as electronics, optical sensing, biomedical sciences, energy storage devices, pollution controlling agents, tissue engineering and cosmetics [4-5]. While using chemicals as reductants for the preparation of AgNPs induces hazardous side effects on our environment. The most commonly used reducing agent, sodium borohydride ($NaBH_4$) releases toxic gases which further lead to corroding the surface of AgNPs [6]. On the other hand, plants are inherent factories of potential bioactive components useful for the NPs production. The polyphenols such as flavanoids, phenolic acids, carbohydrates, polymers and coumarins in plants reduce the silver precursor followed by nucleation, growth, and stabilization of AgNPs [7]. Moreover, the phytochemicals will be essential for moulding the structural and functional properties of the product and having advantages like ease of availability, low production cost, safe to handle, reliable and biocompatible [8-9].

Papaya (*Carica papaya L.*) is a worldwide common nutraceutical, bisexual and nonseasonal flowering fruit plant and the fruit obtained from it is known as the fruit of the common man [10]. Nowadays, *Carica papaya* has been extensively cultivated and consumed in the major fifty-seven nations of the world and India has become the largest producing country and United Nations belongs to the leading importer [11]. Generally, three gender varieties, male, female and hermaphrodite are there in papaya plants [10]. The proteolytic enzymes like papain and chymopapain and the enrichment of antioxidants vitamins A, B1, C and E make the whole plant parts (fruit, leaf, peel, bark, flower, seed and root) beneficial for therapeutic applications [10-11]. Like papaya fruit, from ancient days, papaya flowers also have been consumed as fresh vegetables and used as a medicine on tropical and subtropical continents. The free radical scavenging action of *Carica papaya* flowers has a significant role in modulating our immune system [12]. It has been proven that the phenols, beta-carotene and gallic acid present in papaya flowers help to resist the growth of some cancerous cells in the body. Moreover, it regulates blood pressure and improves the health and functioning of the heart [13-14]. Hence, the potential antioxidant nature of the papaya plant has attracted immense attention in the intensive search for a better biological agent. Even though the other parts of the papaya plant, like fruit, peel, leaf and bark have been utilized in the production of NPs, the flower is yet to explore.

In this study, we have introduced the potent CPE for the first time to fabricate AgNPs via the green method without using any other chemical reagents. The antibacterial property of the as-synthesized CPE-AgNPs was also done against two multidrug-tolerant bacteria *Escherichia coli* and *Staphylococcus aureus* and the evaluation of bacterial growth and suppression were investigated using standard procedures

MATERIALS AND METHODS

Fresh *Carica papaya* male flowers were collected from the farming garden of the National Institute of Technology Calicut, Kerala, India. The collection of the plant material and the research work associated with this complies with relevant institutional, national, and international guidelines and legislation. The one and only chemical used for the synthesis of CPE-AgNPs was the metal precursor salt: Silver nitrate ($AgNO_3$) of analytical reagent grade (98% pure) was purchased from Sigma-Aldrich. The strains of *Escherichia coli* and *Staphylococcus aureus* with culture codes MTCC 443 and MTCC 96 respectively were obtained from the microbial type culture collection and gene bank (MTCC), India.

The formation of biogenic AgNPs was investigated using a UV-1800 Shimadzu spectrometer. The phytochemicals of *Carica papaya* male flower extract which protects the prepared AgNPs via capping mechanism were examined by attenuated total reflection (ATR) mode of a PerkinElmer frontier FTIR spectrometer. The morphological characterization of AgNPs was studied using High-Resolution Transmission Electron Microscope (HRTEM) of FEI Tecnai G2 Spirit Bio-Twin at an operating voltage of 300 KV. For HRTEM analysis, the diluted suspension of CPE-AgNPs was adsorbed onto Cu grid carbon disc and left it for some time to dry before the measurements. The e distribution of prepared NPs and stability of the suspension was evaluated using Malvern Nano ZS particle size analyzer and Microtrac Zeta Check-Particle charge Reader of model: PMX500, respectively. The elemental composition of the nanosuspension was determined by the EDS technique using the Horiba EMAX Energy Dispersive spectroscope.

Preparation of CPE

For the green fabrication of AgNPs, CPE was used for metallic reduction and stabilization processes. The preparation of CPE was done by following the standard procedure with slight modification [15]. In detail, 10 g of fresh flowers were washed and cleaned using double distilled water and crushed in mortar and pestle. The cleaned and weighed flowers were then transferred in a conical flask with 100 ml water and kept on a hotplate at 100 ˚C and left for around 20 minutes. The yield of CPE obtained after extraction was 0.30 % and conducted filtration process at 30 ˚C using syringe-driven filters of pores size 0.22 µM and Whatman's filter paper no.1 grade for removing micro-size impurities. Eventually, the filtrate (pale yellow in color) was then monitored using a UV-Visible (UV-Vis) spectrometer for studying its optical absorption properties and then stored in a refrigerator for future studies. The phytochemical analysis (qualitative) of CPE was evaluated by following the standard procedure mentioned in the existing study [16].

Preparation of CPE-AgNPs

At first, a volume of 3 ml $AgNO_3$ (3 mM) was taken in a container. At room temperature, 300 µl CPE was added in drops to this precursor and the container was gently shaken throughout this mixing process. The formation of CPE-AgNPs was investigated at seven different dilutions of CPE ranging from 0.5 ml to 2 ml (at an interval of 0.25 ml) at the constant Ag ion (Ag^+) concentration mentioned above. A color transformation from pale yellow to brownish-yellow for the suspension was noted as visual confirmation of the formation of CPE- AgNPs.

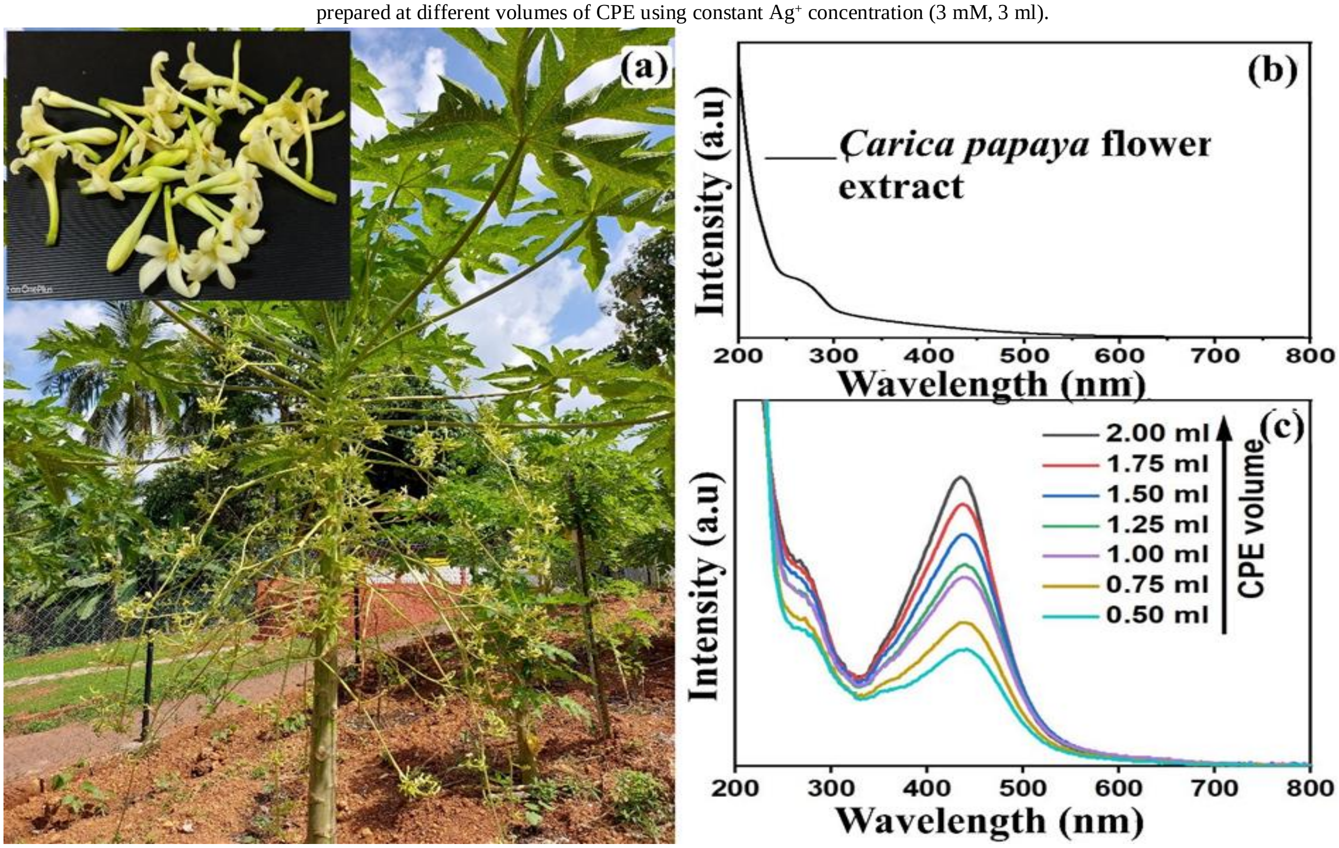
Investigation of Antibacterial Properties of CPE-AgNPs

The antimicrobial efficacy of CPE-AgNPs was investigated against the gram-negative pathogen (*Escherichia coli*) and gram-positive pathogen (*Staphylococcus aureus*), according to the well-diffusion method [17]. These clinical isolate samples were suffused in Luria-Bertani (LB) broth of volume 100 ml and kept for overnight incubation at 37 °C at 100 rpm. 0.1 mg/ml of CPE-AgNPs was made in Milli Q water and the solution was sonicated thoroughly. The bacterial suspension was prepared by adjusting the absorbance to 0.1 at 600 nm using sterile Milli Q water and was uniformly spread on four different LB agar plates for the different organisms. Using a well-borer of 10 mm diameter, two wells were created in which one well was filled with CPE and the other with 100 µl of 100 µg/ml CPE-AgNPs. The incubation procedure was the same as that earlier method and only the duration is changed to one full day. The growth of microorganisms was observed, and the zone of inhibition was studied. The smallest dilution of CPE-AgNPs required to impede the production of microorganisms is defined as the minimum inhibitory concentration (MIC) value. Minimum bactericidal concentration (MBC) is the concentration of CPE-AgNPs having the maximum ability to suppress the lives of pathogens [18].

RESULTS AND DISCUSSION

Carica papaya plant contains a large number of phytochemicals like flavonoids, carbohydrates alkaloids, vitamins, tannins, saponins, glycosides, and different types of minerals like Fe, Ca, Mg, K, Zn, Mn [10-11]. Among the major flavonoid contents like quercetin, kaempferol, and glycosides in CPE, studies reveal that kaempferol 3-(2G-rhamnosylrutinoside) is the major antioxidant flavonoid present in it [19-20]. Fig. 1(a-b) represents a sample photograph of a male papaya tree chosen for this study and flowers collected from it for the preparation of extract, respectively. As shown in Fig. 1a the male papaya tree will be flowering in nature throughout the year and since it doesn't contain an ovary, it won't bear fruits [21]. Compared to female flowers, the male papaya flowers (Fig. 1b) have very small calyx with a long stalk and are clustered in nature. The flowers are always hanging down from stalks. Existing studies reveal the fact that male papaya flowers are phytochemically richer than other gender varieties [21-22].

Figure 1: Photographs of *Carica papaya* male (a) tree (inset showing flowers). UV-Visible absorption spectrum of (b) CPE and (c) CPE-AgNPs prepared at different volumes of CPE using constant Ag^+ concentration (3 mM, 3 ml).



UV-Visible Absorption Study

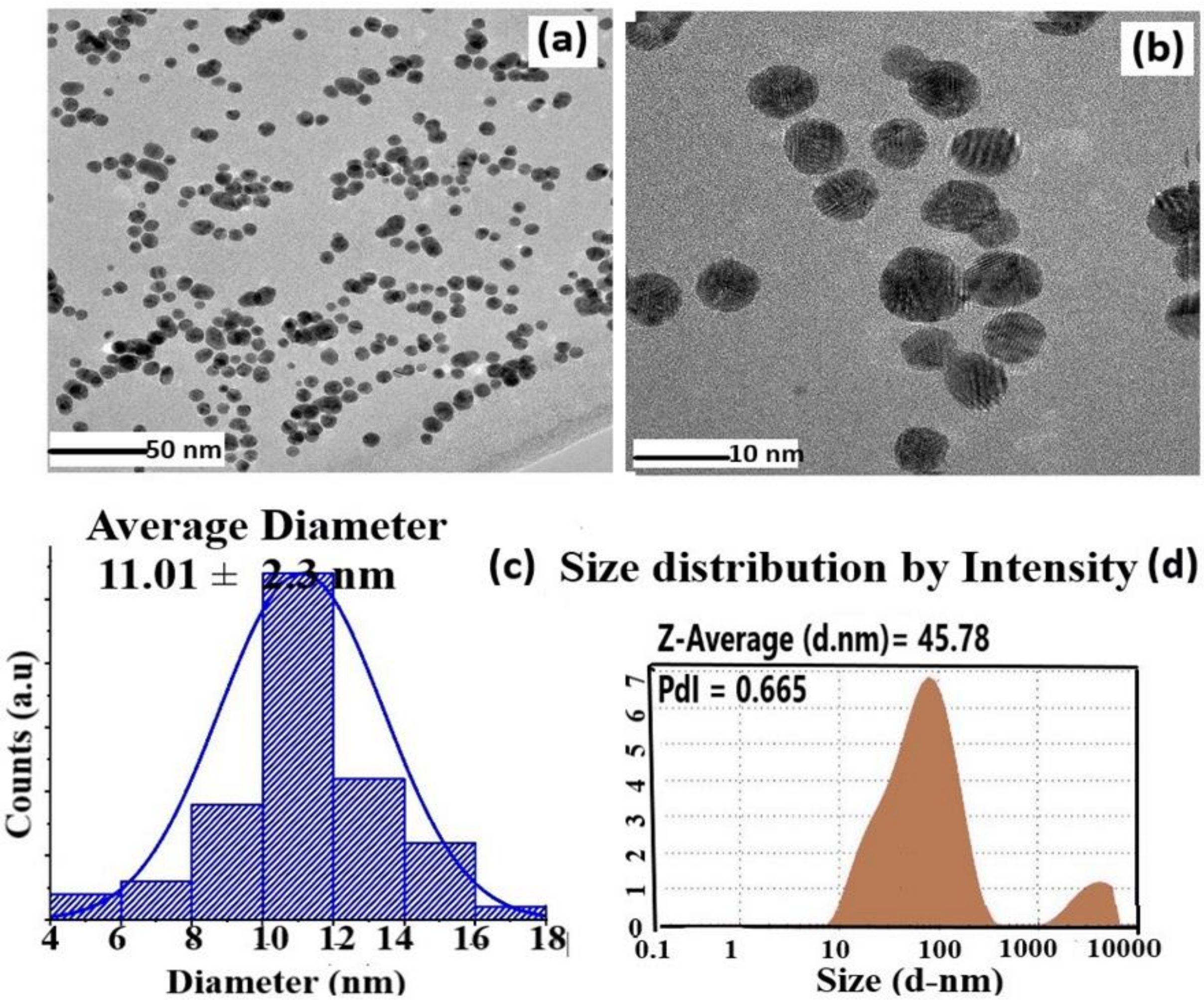
Fig. 1c shows the optical absorption spectrum of CPE in which the absorption region below 300 nm indicates the presence of flavonoids and polyphenols present in it [23]. The reduction of Ag ions to seeds of Ag using CPE requires only 2 minutes, which is visually visible by noticing the brown color of the suspension and its SPR [24-25]. The absorption spectrum of CPE-AgNPs for different dilutions of CPE are depicted in Fig. 1d. The center of SPR band for CPE-AgNPs prepared by adding 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 ml of CPE were 440, 439, 438, 437, 436, 435 and 434 nm respectively. Fig. 1d clearly shows the simultaneous increase in intensity and lowering in full width at half maximum (FWHM) of the UV-Visible spectrum of CPE-AgNPs as the concentration of CPE increases. This indicates the successive reduction and formation of lots of AgNPs with the availability of more bioactive chemicals for reducing the Ag ions for higher concentrations of CPE. Further, the symmetric nature of the SPR band suggests the evolution of monodisperse spherical CPE-AgNPs. Moreover, nano colloid of CPE-AgNPs was checked for its physical stability analysis by monitoring the zeta potential by the means of surface charge measurement. CPE-AgNPs prepared using 2 ml CPE possess a high zeta potential value -65 ± 2.1 mV even after 90 days indicating the excellent stability of as-prepared NPs [26].

This high zeta potential value of CPE-AgNPs reveals better stability provided by the phytochemicals present in CPE as compared to other studies available in the literature. These results imply the role of CPE as reducing and stabilizing agent as well which further proves the CPE as an efficient green alternative for the in-situ preparation of AgNPs.

Morphological and Elemental Composition Analysis

Fig 2a-b represents the TEM images of mono dispersed spherical CPE-AgNPs. The real diameter (averaged value) of the as-prepared CPE-AgNPs evaluated from TEM is 11.10 ± 2.3 nm. The mono dispersed NPs formation indicates the rapid nucleation process during the synthesis process. Hence, the synthesis route proposed in this study is much faster and simple than previously reported [27]. Dynamic light scattering (DLS) provides information on the dynamics of the nano colloidal system by intensity fluctuations of the scattered light. In this case, the fluid layer formed by secondary metabolites of CPE influences the surface structure of CPE-AgNPs.

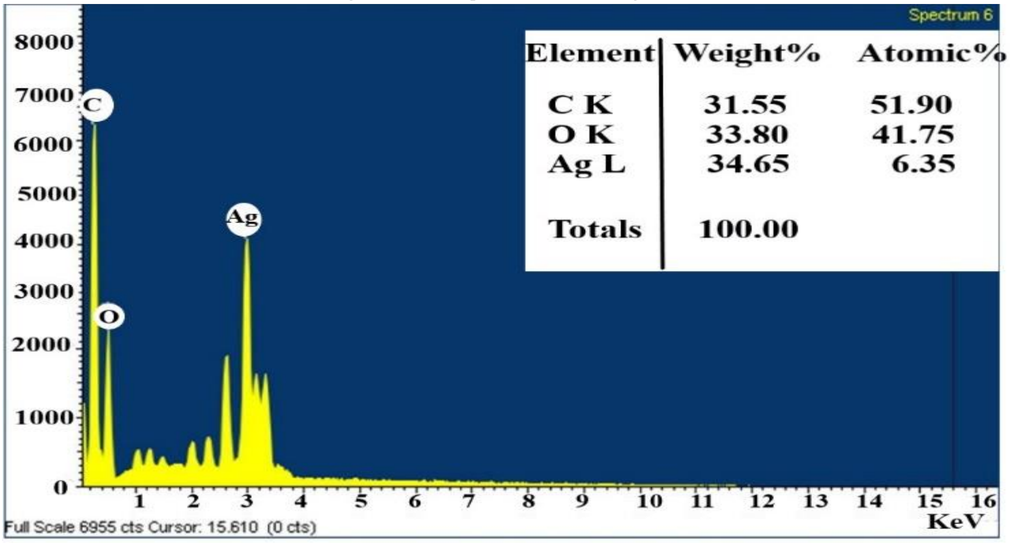
Figure 2: TEM images of CPE-AgNPs (using 2 ml CPE) at scale bar (a) 50 nm (b) 10 nm (inset shows average size distribution histogram). (c) Size distribution by intensity based on DLS study.



Thus, the change in surface structure induces a change in the diameter of NPs being formed [28]. The hydrodynamic diameter of CPE-AgNPs obtained by DLS (Fig. 2c) is 45.78 nm which is higher than that of the real diameter obtained from TEM. In the case of TEM analysis, the presence of a capping agent is excluded hence the diameter obtained from TEM will be always less than that of the diameter calculated using DLS [29].

The chemical constituents present in the CPE-AgNPs were investigated using EDS facility and the result is presented in Figure 3.

Figure 3: EDS spectrum of CPE-Ag NPs.

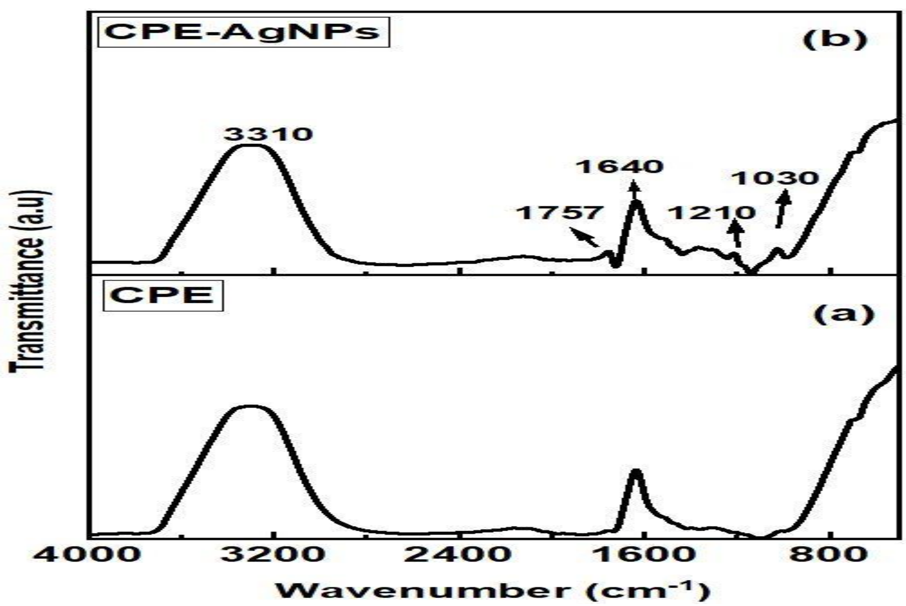


The elemental content of Ag in CPE-AgNPs is 34.65%. The characteristic peak of elemental Ag is confirmed at 3 keV [30]. The involvement of phytochemicals in the reduction and stabilization process during the formation of CPE-AgNPs is evident from the elemental abundance of oxygen and carbon present in the sample under study.

FTIR Vibrational Analysis

The results of FTIR analysis of CPE before the reaction (Fig. 4a) show characteristic peaks at 3310, 2130 and 1640 cm^{-1}. The widespread uptake at around the high wavenumber region (3310 cm^{-1}) of the spectrum is assigned to the OH stretching vibrations of free alcohols, carboxylic acids, water content and flavonoids in CPE [24-27]. The medium broad bands at 2130 cm^{-1} also indicate combination bands of water. C=C bond formation is evident from the sharp feature of band obtained at 1640 cm^{-1} [27]. The vibrational spectrum of CPE-AgNPs shows additional peaks at 1757, 1210 and 1030 cm^{-1} belonging to C=O stretching, C-H bending, and C=C deformation respectively. The emergence of this sharp peak at 1757 cm^{-1} indicates the involvement of carbonyl or ketone moieties of flavanoids present in CPE while forming NPs [27]. Bending vibrations of the C-H group and C=C deformation at 1210 and 1030 cm^{-1} attributes the presence of polyphenols and flavonoids on the surface of CPE-AgNPs [24-27].

Figure 4: FTIR vibrational spectra of (a) CPE and (b) CPE-AgNPs.

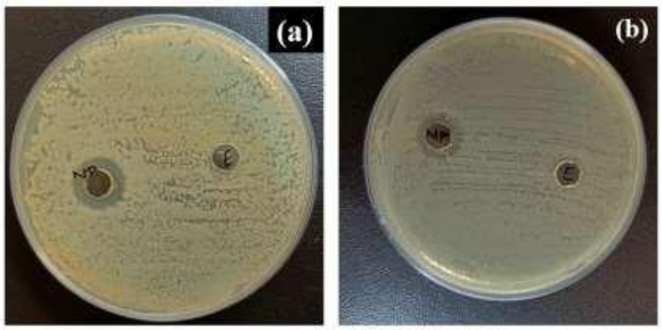


Antibacterial Activity of CPE-AgNPs

The application of green-reduced AgNPs as a therapeutic agent against several pathogens has been widely accepted and explored since we recognized the relevance practicing of green chemistry protocols [26-27,31]. Well diffusion method, MIC and MBC are standard microbiological tests followed by researchers to implement and study the antibacterial activity of NPs [18]. The zone of inhibition obtained for CPE and CPE-AgNPs for *Escherichia coli* and *Staphylococcus aureus* is depicted in Fig. 5a and 5b, respectively and tabulated in Table 1.

The preliminary well diffusion method shows that there is no visible antibacterial activity shown by CPE alone. Whereas the sample CPE-AgNPs showed a maximum inhibition zone for both gram-positive and gram-negative bacteria in this study. The biggest zone of inhibition 15 mm was obtained for *Escherichia coli* compared to 14 mm in case of *Staphylococcus aureus*.

Figure 5: Well diffusion assay of CPE-AgNPs (NP) and CPE (E) on the strain of (a) *Escherichia coli* and (b) *Staphylococcus aureus*.



Investigation on MIC and MBC of CPE

Herein this report, we have investigated the application of green fabricated CPE-AgNPs as an antimicrobial agent on Gram-negative bacteria and Gram-positive bacteria on an agar plate and liquid medium. Moreover, CPE-AgNPs exhibit excellent bactericidal activity against the tested microorganisms. The MIC and MBC of CPE-AgNPs evaluated against *Escherichia coli* is 18 & 21 mg/ml and that of *Staphylococcus aureus* are 7 & 10 mg/ml, respectively (as shown in Table 1).

Table 1: Results of antibacterial property of CPE-AgNPs on bacterial cultures.

	Pathogen	Inhibition zone of CPE-AgNPs (mm)	MIC of CPE-AgNPs (mg/ml)	MBC of CPE-AgNPs (mg/ml)
CPE- AgNPs	*Escherichia coli*	15	18	21
	Staphylococcus aureus	14	7	10

Hence, these results clearly illustrate the better antimicrobial activities of our CPE-AgNPs via inhibiting the growth and bactericidal effect as well. Moreover, as prepared green synthesized CPE-AgNPs can be an alternative for developing silver-containing medical devices like urinary catheters against UTIs [30].

CONCLUSION

We have successfully synthesized highly stable, eco-friendly, and monodispersed AgNPs via a completely green and facile in-situ synthesis route. The aqueous extract of the male papaya flower is used as a reducing and stabilizing factor. As prepared CPE-AgNPs are uniformly dispersed, and have a geometry of sphere with a real diameter of 11.10 ± 2.3 nm. The as-synthesized CPE-AgNPs possess an excellent zeta potential value of -65 ± 2.1 mV. These CPE-AgNPs further show high antibacterial activity against both the gram-negative and positive bacterial strains and can be used for developing bacterial-resistant silver-containing urinary catheters against UTIs.

ACKNOWLEDGEMENTS

The financial support for this work was granted from Faculty Research Grant [NITC/DEAN(R&C)/FRG/2018- 19/3], NIT Calicut, Kozhikode - 673601, Kerala, India. We thank, Dr. Muniyandi Sankaralingam (Department of Chemistry), and Dr. N. Sandya Rani (School of Material Science) for the technical support for UV-Vis studies. The author expresses sincere thanks to Dr. S. Bhuvaneshwari (Chemical Engineering) for providing research facilities for zeta potential measurements.

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