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Full Length Article

Green Synthesis of Ag Nanoparticles from Aqueous Extracts of Leaves and Fruit of *Casuarina equisetifolia* against *Candida albicans* and other Clinical Isolates

Mahmoud Moustafa[1,2*], Ahmed Al-Emam[3,4], Mahmoud Sayed[5,6], Saad Alamri[1,7], Huda Alghamdii[1], Ali Shati[1], Sulaiman Alrumman[1], Mohamed Al-Kahtani[1], Eman Khalaf[8], Thanaa Maghraby[2] and Hanan Temerk[2]
[1]*Department of Biology, College of Science, King Khalid University, 9004, Abha, Kingdom of Saudi Arabia*
[2]*Department of Botany and Microbiology, Faculty of Science, South Valley University, Qena, Egypt*
[3]*Department of Pathology, College of Medicine, King Khalid University, Abha, Saudi Arabia*
[4]*Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Mansoura, University, Mansoura, Egypt*
[5]*Physics Dep., Faculty of Science, King Khalid University, P. O. Box 9004, Abha, Saudi Arabia*
[6]*Physics Department, Faculty of Science, Al-Azhar University, P. O. 71452, Assiut, Egypt*
[7]*Prince Sultan Bin Abdulaziz Center for Environmental and Tourism Research and Studies –King Khalid University, Kingdom of Saudi Arabia*
[8]*Department of Microbiology and Immunology, Faculty of Pharmacy, Damanhour University, Damanhour, Egypt*
[*]For correspondence: mfmostfa@kku.edu.sa; mfmostfa@kku.edu
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Abstract

The prevalence of the infectious diseases caused by Candida species and other human pathogenic microbes has led to the discovery of some natural agents against multidrug resistant microbes. Therefore, this research was designated to determine the capability of synthesized nanoparticles (NPs) from aqueous extract of leaves and fruits of *Casuarina equisetifolia* as anticandidal and antibacterial against some microbes. Silver NPs (AgNPs) were successfully gained from aqueous leaves and fruit extracts of *C. equisetifolia* which is defined on the basis of UV–visible spectroscopy, X-ray diffraction analysis and scanning electron microscope. The results showed that leaves and fruits extracts of *C. equisetifolia* acts as an excellent capping agent. XRD based on the FWHM analysis showed that AgCl and Ag had an average NPs size of 90.97 nm and 71.28 nm, respectively for fruits and 15.33 nm and 14.01 nm, respectively for leaves. UV–visible spectroscopy showed a maximum absorbance at 442 nm for fruits and 433 nm for leaves. SEM showed that the size of NPs from leaves lied in between of (30 to180 nm) and for fruits (70 to 250 nm). *Candida albicans* was severely affected by NPs of leaves with inhibition zone (3.03± 1.61 cm) and NPs of fruits had (1.37± 0.15 cm) inhibition zone. Nanoparticles from leaves exhibited maximum activity against *P. mirabilis* (3.52± 0.13 cm) and low activities against *M. luteus* (1.50±0.18 cm) inhibition zones. In conclusion, these eco-friendly synthesized AgNPs from leaves and fruits of *C. equisetifolia* could be used as competitive alternative natural drugs than conventional synthetic chemicals.

Keywords: *Casuarina equisetifolia*; AgNPs; Anticandidal; Antibacterial; Aqueous extracts

Introduction

The construction of functional molecule at micro level is of great interest for nanotechnology techniques. Nanomaterials (NPS) are used extensively in the area of medicine for the purpose of drug delivery, diagnosis, treatment of cardiovascular disorders, wound healing and production of antimicrobial agents. They exhibit unique physico-chemical properties, which are not observed in either single molecules or bulk metals itself (Dauthal and Mukhopadhyay 2016). They are used for multiple purposes for industrial and in medicinal applications (Thakkar *et al.* 2010; Linic *et al.* 2015). Silver nanoparticles (AgNPs) were more concerned than other metallic nanoparticles (MNPs) because of their special properties such as magnetic, optical polarization, electrical conductivity (EC) and prominent antimicrobial activity (Evanoff and Chumanov 2005). Considering the presence of various metals in nature, only some of them, such as silver, gold, palladium and platinum, are extensively synthesized in nanostructured form (Yoon *et al.* 2010; Yang *et al.* 2017). Three different methods of synthesis for NPs developed are chemical, physical and green synthesis (Oves *et al.* 2018; Saratale *et al.* 2018).

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Physical methods require expensive equipment, high temperature and high pressure. For the synthesis of nanoparticles using chemical processes, harmful substances are used, which can cause significant harm to the atmosphere and to living organisms. Due to these disadvantages, the use of chemical and physical methods is limited and gradually is replaced by green synthesis, which is a more environmentally friendly and cheaper method. Green synthesis in all process is similar to chemical reduction where costly chemical reducing agent is substituted by a natural product extract, such as leaves, root or fruits are applied for NPs synthesis of metal or metal oxide (Hussain *et al.* 2016). Natural product NPs are considered to be environmental friendly and safe (Jayaseelan *et al.* 2012; Gopinath *et al.* 2014), free from environmental pollution (Chandran *et al.* 2006; Huang *et al.* 2007), cheaper (Mittal *et al.* 2013) and ideal for mass manufacturing (Iravani 2011). In addition, biologic development of NPs makes it possible to recycle expensive metal salts such as silver and gold contained in waste streams (Wang *et al.* 2009).

Plants, bacteria, fungi, algae, etc. are commonly used for green nanoparticle synthesis (Chen *et al.* 2015; Khan *et al.* 2016; Agarwal *et al.* 2017). The ability of pathogenic candida and bacteria to resist against various drugs is a major obstacle in medical practice that restricts the effectiveness of such normal medications (Quelemes *et al.* 2013). Such disadvantages give researchers enormous opportunities to create new substances, such as AgNPs, to counter such problem.

Casuarina equisetifolia L. is predominantly a monoecious species belonging to the Casuarinaceae family (Binggeli *et al.* 1999). It is planted as ornamental in the street side or as a fence around house or may be growing wildly in abandoned area. It is found in many climatic regime, usually grow near sea level but succeeding under cultivation at heights up to 1,400 meters and can grow under saline environments (Jensen 1995; Garrity *et al.* 2006). The plant contains several phytochemical components such as glycosides, quercetin, triterpenoids, tannins, rutin and casuarine, catechol derivatives etc. (Ramanathan *et al.* 2012). The existence of natural secondary metabolites shows the tree species as important source for use as an astringent, diuretic agent for coughing, diarrhea, beri-beri, colic and as toothache. Biological properties such as hypoglycemic anticancer activity of this tree species have been characterized (Han 1998; Parekh *et al.* 2006).

Candidiasis is caused by various *Candida* species and considered as one of the most prevalent fungal infections to humans, especially by *C. albicans*. There are no reports till date about the biosynthesis of (AgNPs) by applying aqueous extract of leaf and fruit of the Casuarinaceae family. Herein, an effort has been made to examine NPs from leaf and fruit extract of the *C. equisetifolia* against *C. albicans* isolates and against a range of human pathogenic microbes as potential drugs for pharmaceutical application. Also, in the present study NPs from leaf and fruits had been characterized using various techniques. This research was based on a rather simple hypothesis that plant aqueous extracts contain a group of polar compounds rather than secondary metabolites. Thus, depending on that fact, simple preparation of AgNPs from leaves and fruits of *C. equisetifolia* have been relaying on water extraction.

Materials and Methods

Collection of plant materials

Leaves and fruits samples of *C. equisetifolia* were collected from Abha region, KSA during the month of May in 2019 (Latitude: 18.2114609; Longitude: 42.4999752). The obtained materials were thoroughly rinsed in flowing tap water to remove adhesive particles, and then with distilled water until no traces of foreign material remained.

Preparation of plant extract

A 30 g of fresh leaves and fruits of *C. equisetifolia* was crushed thoroughly with a blender (Philips, Germany) to a homogeneous mass with the addition of 30 mL of DW (1 g/mL). Then extract was filtered using Whatman No 1 filter paper, and the supernatant was collected and stored at 4°C for further analysis.

Synthesis of silver nanoparticle

Silver nitrate reagent (0.5 m*M*) was made using deionized water, to synthesis silver nanoparticle from leaves and fruits extract of *C. equisetifolia*. Ten mL from each was added to 90 mL of 0.5 m*M* $AgNO_3$ (Saranya and Gowrie 2016). The mixture was heated at 70°C for 3 min. The reduction of aqueous silver ions by both extract to form stable AgNPs was noted with the formation of yellow, brown and then blackish colored solution (Kirthika *et al.* 2014).

Characterization of silver nanoparticles

UV-Vis Spectrophotometer: Reduction of Ag^+ ions was spectrophometrically monitored using double-beam UV-Vis spectroscopy (Hitachi, U-3010) by dissolving a small aliquot of the reaction solution into distilled water. The wavelength of 350–500 nm was measured and the peak was recorded.

Powder X-ray Diffraction (XRD): Powdered silver nanoparticles were mounted on a microscopic slide and air dried at 24°C overnight. The crystalline form, phase detection and grain size using XRD (Shimadzu, 6000 Diffractometer, Japan) were achieved.

Scanning electron microscope (SEM): The AgNPs morphology images from leaves and fruits extracts from *C. equisetifolia* were studied via SEM (JSM-7500 F; JOEL-Japan). Some drops from the sample colloidal solution were dried at 60°C in a high stability furnace on microscopic sheet and then observed.

Anticandidal and antibacterial activities

In vitro antimicrobial behavior of processed silver nanoparticles using leaves and fruit extracts of *C. equisetifolia* was investigated. *Candida albicans* and other six human pathogens *viz.*, *Proteus mirabilis*, *Psedomonas aeruginosa*, *Staphylococcus aureus*, *Shigella flexneri*, *Klebsiella pneumoniae* and *Micrococcus luteus* were used for anticandidal and antibacterial studies, which were obtained from the Department of Microbiology, King Khalid University, Saudi Arabia. These microbes were grown in nutrient broth for 48 h. Agar well diffusion method was used to screen the antibacterial and anticandidal activities of NPs gained from aqueous extract of leaves and fruits of *C. equisetifolia* (Daoud *et al.* 2019). Potato Dextrose Agar and nutrient agar media were prepared for *C. albicans* and for bacterial strains respectively and from each 20 mL were poured into sterilized Petri dish. Upon solidification, 1 mL from fresh candida and bacterial culture was pipetted in the center of sterile Petri dish and swapped uniformly upon the surface of solid media using Sterile Cotton swabs. In each plate two wells were punctured by autoclaved cork borer (6 mm in diameter) into agar plates containing inoculums. Then 100 μL AgNPs of each extract was applied to the respective well. Plates were refrigerated for 30 min to allow the extracts to spread well into the agar. The plates were then incubated at 37°C for 48 h. Anticandidal and antibacterial activities were detected by measuring the inhibition zone (including the well diameter) that occurred during the incubation period. DMSO at a concentration of 10% was employed as a negative control and Cefoxitin (30 mcg) disc was used as positive control. Each plate was examined after incubation and the diameters of the inhibition zone were determined using the millimeter ruler. A region of inhibition of about 9 mm or more around the holes was defined that the extracts have antimicrobial substance (Alamri and Moustafa 2012). All experiment was performed in three replicates.

Statistical analysis

Results were subjected to one-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) *Post Hoc* test of three replicates ± standard deviation (SD). Differences between means in relation to positive control were considered significant at p-value < 0.05.

Results

Antimicrobial activity

The antimicrobial activity behavior of the biosynthesized AgNPs is shown in Fig. 1. There was remarkable inhibition activity of AgNPs against *C. albicans*, Gram-positive and Gram-negative bacteria. The maximum inhibitory effects of AgNPs were observed against all tested microbial strains

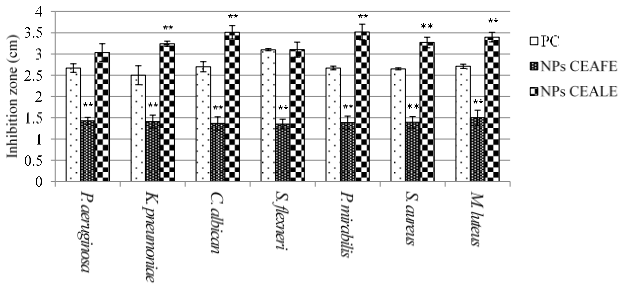

Fig. 1: AgNPs anticandidal and antibacterial activities from leaves and fruits of *C. equisetifolia* aqueous extracts against *C. albican*, *P. aeruginosa*, *K. pneumoniae*, *S. flexneri*, *P. mirabilis*, *S. aureus* and *M. luteus*. PC, positive control. Significant differences (*$P \leq$ 0.05; **$P \leq$ 0.01), between treatments in relation to PC ± SD of the mean for n = 3

when prepared from *C. equisetifolia* leaf extract. While AgNPs from fruits extract of *C. equisetifolia* showed low antimicrobial activities against all tested microbial strains. For *C. albicans* the zone of inhibition from AgNPs of aqueous fruits was (1.37±0.15cm). With an inhibition zone between 1.5–1.35 cm, it was considered that all the tested strains were susceptible to AgNPs from fruits extract of *C. equisetifolia*, which implied that the extract have antimicrobial properties (Nascimento *et al.* 2000; Alamri and Moustafa 2012). NPs of aqueous fruits extract showed maximum antibacterial activities against *M. luteus*, followed by *P. aeruginosa* and moderate activity against *S. aureus*, *P. mirabilis*, *K. pneumoniae* and *S. flexneri* (Fig. 1). AgNPs from aqueous leaves extract of *C. equisetifolia* showed potent antimicrobial activities against all tested microbial strains more than fruits extract between 61.06 and 52.97%. *C. albicans* was severely affected by AgNPs leaves extract with a zone of inhibition of 3.03±1.61 cm. AgNPs of aqueous leaves showed maximum antibacterial activities against *P. mirabilis* (3.52±0.13 cm), followed by *K. pneumoniae* (3.24±0.06 cm) and *M. luteus* (3.40±0.14 cm). *S. flexneri*, *P. aeruginosa* and *S. aureus* demonstrated susceptibility in the range between (3.27±0.11 cm and 3.03±0.21 cm with AgNPs from leaves extract. The percentage susceptibility differences among various bacterial strains between AgNPs of leaves and fruits extracts was; *P. mirabilis* > *S. aureus* > *S. flexneri* > *K. pneumoniae* > *M. luteus* > *P. aeruginosa*. *C. albican* was very sensitive to $AgNP_S$ from leaves and fruits extract as the maximum differences between them was found to be 61.06%. DMSO showed no antimicrobial activities against all tested microbes while positive control inhibited microbial growth in the range between 3.10 and 2.55 cm increased as compared to AgNPs of fruits extract which was 3.1 to 1.93%. Efficacy of AgNPs from leaves extract was higher than positive control by 39.96% against *P. mirabilis* to 15.48% against *P. aeruginosa*. Remarkable differences were also found against *C. albicans* by AgNPs of leaves extract of *C. equisetifolia* being more than positive control (Fig. 1).

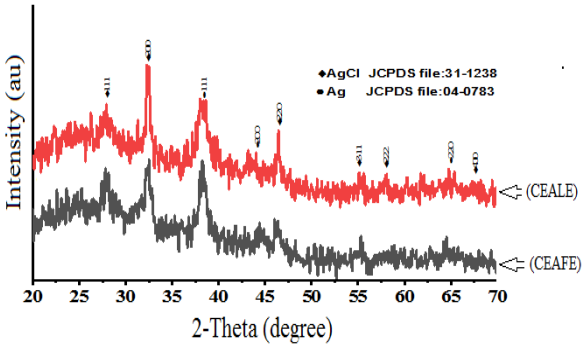

Fig. 2: X-ray diffraction pattern of AgNPs from *C. equisetifolia* aqueous leaves extract (CEALE) and aqueous fruits extract (CEAFE)

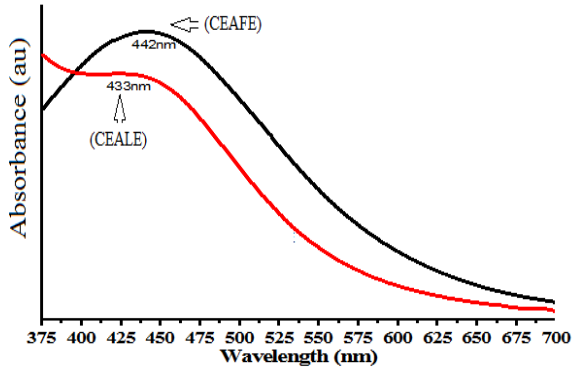

Fig. 3: The UV/Vis absorption spectra of AgNPs from *C. equisetifolia* aqueous leaves extract (CEALE) and aqueous fruits extract (CEAFE)

NPs characterizations using XRD

Fig. 2 shows the XRD pattern of NPs from *C. equisetifolia* leaves aqueous extract (CELAE) and fruits aqueous extract (CEAFE). There was similarity to some extent between the positions of the diffraction peaks for both patterns. For CEAFE, the diffraction peaks were found at 2-theta values at 38.20˚ (111), 44.40˚ (200), and 64.55˚ (220), corresponded to the crystal planes of the face-centered cubic(fcc) Ag (JCPDS card No. 04-0783); whilst, five additional diffraction peaks appearing at 2-theta values of 27.78˚ (111), 32.49˚ (200), 46.37˚ (220), 54.47˚ (311), 57.31˚ (222) and 67.10˚ (400) correspond to the crystal planes of the face-centered cubic (fcc) AgCl (JCPDS file:31-1238). Similarly, for CELAE extraction, the diffraction peaks at 2-theta values at 38.08˚ (111), 43.93˚ (200), and 64.74˚ (220), correspond to the crystal planes of the face-centered cubic(fcc) Ag (JCPDS card No. 04-0783); but, five additional diffraction peaks appearing at 2-theta values of 27.81˚ (111), 32.37˚ (200), 46.41˚ (220), 55.10˚ (311), 57.96˚ (222) and 67.22˚ (400) correspond to the crystal planes of the face-centered cubic(fcc) AgCl (JCPDS file:31-1238). These data confirmed that Ag/AgCl nanoparticles are synthesized via both extraction of plant. Other peaks occured in the XRD pattern that could be due to the crystallization of bio-organic particles on the surface of the AgNPs. Furthermore, the average crystal size (Table 1) was determined by the Debye-Scherrer formula: $D=(0.9\lambda)/(\beta\cos\theta)$, where D is the average size of the crystals, λ is the wavelength of radiation, β is the full width at half the maximum height (FWHM) and θ is the position of the maximum diffraction peak. Based on the FWHM of the diffraction peak (200) of the AgCl crystals and (111) the diffraction peak of the Ag crystals, the AgCl and Ag average nanoparticle size was determined and found as 90.97 and 71.28 nm, respectively for CEAFE, whereas D was reduced to 15.33 and 14.01 nm for CELAE.

UV-vis spectrophotometer and SEM analysis

In the present study, the solution of $AgNO_3$ was immediately turned into blackish after the addition of *C. equisetifolia* leaves and fruit extract as reducing agents in all samples showing the formation of AgNPs. Fig. 3 shows the typical UV spectra of the reaction solution with a localized surface plasmon resonance (SPR) of about 442 and 433 nm for AgNPs synthesized using extract of (CEAFE) and (CEALE), respectively, which strongly indicated the formation of AgNPs. Moreover, there was SPR semi-flat peak, which resulted from the presence of Ag/AgCl NPs.

SEM images of the synthesized AgNPs extracted by CEAFE and CEALE respectively are shown in Fig. 4. The CEALE AgNPs showed ultrafine rounded to sub-rounded nano-particles of average size 30–180 nm dispersed on the surface of bio-organic sheets as a residue material. However, CEAFE AgNPs exhibited different particles-morphologies and the particle size was between 70-250 nm relatively more than those of CEALE.

Discussion

Based on gained NPs antimicrobial analyses against tested microbes, it was noticed that the mode of action included bactericidal effect against bacterial strains and candidcidal against *C. albicans* of AgNPs were most possibly due to the binding of AgNPs to the cell wall and the production of free radicals (Pirtarighat *et al.* 2019). Our explanation why NPs prepared from leaves more potent than fruits this probably due to leaves have more chemicals than fruits. In addition, it was reported that AgNPs could disrupt the permeability of the membrane by entering the cell membrane and inducing intracellular ATP leakage and finally causing the cell death (Hajipour *et al.* 2012; Ajitha *et al.* 2014). Positively charged ions from Ag^+ seem to have a strong tendency to act with phosphorus and sulfur in biomolecules such as DNA and RNA, so that such attachment of small NPs from *C. equisetifolia* leaves leads to the interruption of the roles of DNA and RNA in the cell (Hajipour *et al.* 2012).

Table 1: Experimental diffraction angles, FHWM, d-spacing, diffraction planes and particles size of silver (Ag) and silver chloride (AgCl) extracted by aqueous extracts from fruits (CEAFE) and leaves (CEALE) of *C. equisetifolia*

Sample	Crystalline phases	Diffraction angle (degree)	FHWM in radians	Lambda (nm)	d-spacing (nm)	Diffraction plane	Particle size (nm)
CEAFE	AgCl	38.20	0.001587	0.154	0.275	(200)	90.97
	Ag	32.49	0.002058	-	0.235	(111)	71.28
CEALE	AgCl	32.37	0.00942	0.154	0.276	(200)	15.33
	Ag	38.08	0.010467	-	0.236	(111)	14.01

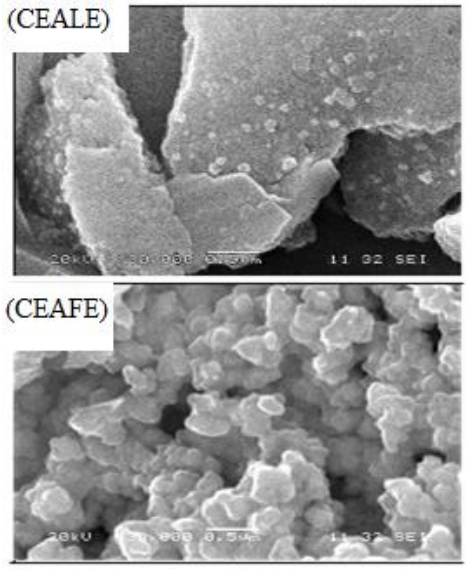

Fig. 4: SEM of AgNPs from *C. equisetifolia* aqueous leaves extract (CEALE) and aqueous fruits extract (CEAFE)

It has been reported that the smaller the particle size of the AgNPs, the more microbial cell damage will occur, due to oxidative stress response due to reactive oxygen species (ROS). Again, ROS caused DNA damage, and the rupture of the cell membrane of the microbial strains. In agreement with our findings that nanoparticles have a greater surface area available for contact that improves the bactericidal and candidcidal effect than large particles (Raffi *et al.* 2008). Previous works showed that various NPs from plant extracts had promising candidcidal effect as Zn nanoparticles combined with fluconazole could inhibit the vaginal candidiasis (Nazari 2020).

As the particles size increase, the position of the absorption peak is shifted to the long wave, and the larger the particle size, the more visible the phenomenon of red transformation. By altering extraction using *C. equisetifolia* aqueous leaves extract to aqueous fruits resulting in a blue shift from the SPR range from 442 to 434 nm implied that smaller NPs were found there. Small AgNPs may be due to higher nucleation rate with the improved reduction process which supports more potent antimicrobial activities (Khan *et al*. 2013; Dong *et al.* 2019).

Agglomeration of AgNPs in the form of nanoclusters may be due to the effect of plant extract during sample preparation for SEM. Again, this supports the view that tiny size of nanoparticles may assist in easy penetration of microbial cell wall and destroy various organelles. Biosynthesized small NPs have been identified as being able to inhibit/destroy microbial cells (Singh *et al.* 2019). In agreement with previous experimental results (Saranya and Gowrie 2016), this study suggested that the application of AgNPs synthesized from leaves and fruits aqueous extract of *C. equisetifolia*, could be used as an effective antimicrobial agent.

Conclusion

Using aqueous extract of *C. equisetifolia* yielded stable AgNPs as confirmed by UV–Vis, XRD and SEM techniques. Overall, AgNPs from aqueous leaves and fruits extracts from *C. equisetifolia* possessed antibacterial and anticandidal activities as they could inhibit the growth of selected *C. albicans* and bacterial strains. AgNPs from leaves of *C. equisetifolia* had more potent antimicrobial activity than fruits which may be used as natural agent to control the growth of many pathogenic microbes. More research is needed to characterize NPs from *C. equisetifolia* in order to find better antimicrobial activity.

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Author Contributions

MM, MS, EK, TM and HT planned and doing the experimental works, AA and HA interpreted the results and MM, MS, MA, EK, TM, SA, SUA, AS made the write up, analyzed the data and edited the review.

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