

Original Research Article

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Green Synthesis of Copper Nanoparticles Using *Momordica charantia* Fruit Extracts and Evaluation of Their Anti-Microbial Efficacy

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The development of nanotechnology interests the researchers for synthesis of nanoparticles with various bio-applications. The green synthesis of copper nanoparticles using *Momordica charantia fruit* extract acts as both reducing and capping agent. The biosynthesized CuNps were characterized by using UV-Vis analysis, Dynamic Light Scattering (DLS), Fourier Transform Infrared analysis (FTIR), X-ray diffraction analysis (XRD), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) analysis. The synthesized CuNps were in spherical structure with an average size of particle size of 86.07nm. The Antimicrobial activity of the CuNps was established using disc diffusion and agar well diffusion method. The assay showed that CuNps can be a potent antimicrobial agent against *Staphylococcus aureus*, *Pseudomonas aerogenosa* and *Escherichia coli* with no inhibition against *Aspergillus flavus*.

Introduction

Recent research areas have been channeled to the development of anti-microbial agents from natural sources as there is an increasing trend in the emergence of resistance to synthetic anti-microbial drugs due to not only poor quality of drugs, patient non-compliance and irrational use of antimicrobial agents, but also to spontaneous mutations within the microbial populations. Primarily, multiple drug resistance (MDR) developed due to the indiscriminate use of commercial antimicrobial drugs to treat such infectious

diseases (Dey *et al.*, 2010). Owing to the side effects and the resistance that pathogenic micro organisms build against antibiotics as well diseases arising from oxidative stress, drastic measures should be adopted to control the use of anti-microbial agents. Many scientists are paying attention to medicinal plants with biologically active polyphenolic compounds isolated from plant species which possess anti-microbial, antioxidant and nutraceutical properties (Gin and Rigalleau, 2000).

An emerging area of science used to optimize the efficiency of bioactive compounds responsible for the medicinal properties of plants is “Nanotechnology” as it’s a precise and most advanced method of synthesizing highly stable bioactive compounds (Singh *et al.*, 2010). Biological synthesis of nanoparticles from plants extracts slows enzyme kinetics for catalytic activity and offers better manipulation, control over the crystal growth and stability (Prasanth *et al.*, 2011).

Now-a-days scientists are expanding interest in metal nanoparticles (zinc, copper, gold, silver, iron, gold and aluminum) as they provide superior material properties with functional versatility. Plant materials are also used as nanofabricators to promote green synthesis which is less expensive and less toxic (Singh, 2010).

Very few studies have been reported on the use of bitter gourd as a nanofabricator. Pandey (2012) reported the use of *M. charantia* extract for the facile synthesis of ultra-stable gold nanoparticles. Similarly, the green synthesis of silver nanoparticles in bitter gourd extract has been demonstrated by Bhor, 2014. For the first time, the present study reports the use of *Momordica charantia* for the biosynthesis of CuNps and its antimicrobial properties.

Materials and Methods

Sample preparation

Dried sample of *Momordica charantia* was subjected to exhaustive extraction by cold maceration in ethanol for 72 hours in conical flasks that were sealed to avoid evaporation. The slurry obtained was centrifuged at 3,000 rpm for 10 minutes and filtered through Whatman No.41 filter paper. The clear filtrate of 10 ml was taken and mixed with 90 ml

aqueous solution of 1.0×10^{-3} M copper nitrate and incubated at room temperature for 24 hrs. The color change of copper nitrate indicates the formation of CuNps due to reduction of copper ion from Cu^{+2} to Cu. The samples were then centrifuged at 4000 rpm for 15 min to get a clear supernatant at room temperature.

Characterization studies of CuNps

UV – Visible Spectrophotometer was used to record the localized surface plasmon resonance of copper nanoparticles at 200 – 800 cm^{-1} . The size and morphology were examined using Dynamic Light Scattering (DLS), Scanning electronic Microscopy (SEM) and Transmission Electron Microscopy (TEM). FTIR spectrum was recorded in mid IR region in the range of 400 – 4000 wavenumber (cm^{-1}). The structure of the nanoparticles was obtained from X-ray diffraction (XRD) technique.

Determination of antimicrobial activity

The effect of ethanol extract of *M. charantia* and its derivative copper nanoparticles on bacterial strains like *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* and fungal strain of *Aspergillus niger* were assayed by agar well diffusion method and disc diffusion method as described by Pooloth, 2013.

Statistical analysis

All of the data from three independent replicate trials were subjected to analysis using Statistical package: Statistics 8.0. The data are reported as the mean + SD and significant differences between mean values were determined with one way analysis of variance (RBD) (Snedecor and Cochran, 1983).

Results and Discussion

Characterization studies

U.V Visible Spectrophotometer

The absorption spectrum was recorded for the sample in the range of 200–800 nm. The spectrum showed the absorbance peak at 235 - 250 nm corresponding to the characteristic localized surface plasmon resonance (LSPR) band of CuNps. The overall observations suggest that the bio-reduction of Cu^{+2} to Cu was confirmed with UV–Visible spectroscopy (Figures 1 and 2).

Dynamic light scattering and zeta potential measurements

DLS is a technique used to determine the size, size distribution profile and poly disparity index of particles in a colloidal suspension. The measurement results show the DLS and zeta potential to be 88 nm and 30.0 mV respectively (Figures 3 and 4). The significance of zeta potential is that its value can be related to the stability of colloidal dispersions and also indicates the degree of repulsion between adjacent and similarly charged particles in dispersion (Sindhura *et al.*, 2014). The result shows the extracts were stable at room temperature.

Scanning Electron Microscope and TEM

SEM analysis was used to provide information about the morphology and size of the synthesized copper nanoparticles. Figure 5 shows that the nanoparticles formed were spherical in nature and were intactly coated with the material. The SEM micrograph also revealed the size of the nanoparticles was less than 100 nm (average value of 87.06 nm) which confirms the feasibility of synthesizing copper nanoparticles using *Momordica charantia* as nanofabricator.

FT-IR measurements

The FTIR spectroscopy (Figure 6) of the ethanol extract coated with CuNps showed prominent peaks at 3354, 1656, 1653, 1383, 1165 and 426 cm^{-1} were due to O-H stretching, C=N stretching, C=O stretching, C-H group (aromatic), C–N stretching (aliphatic amines) which were skeletal vibrations respectively. The majority of the IR bands were characteristic of triterpenes, proteins, steroids, carbohydrates, alkaloids and other compounds present in the solution. In particular, the broad and intense absorption peak at around 3354 cm^{-1} corresponded to the OH stretching vibrations of phenolic compound like gallic acid (David *et al.*, 2014).

X-Ray diffraction

XRD is a very important method to characterize the structure of crystalline materials and used for the lattice parameters analysis of single crystals or the phase texture and stress analysis of sample. XRD pattern of synthesized CuNps from ethanol extract is shown in figure 7. The sample demonstrated a good crystallinity level with diffraction angles of 32.43, 38.6, 44.80, 64.90, and 77.13 which correspond to the characteristic of face centered cubic of copper lines indexed at (111), (200), (200), (311) and (222).

Antimicrobial properties

The phytochemical profile of *M. charantia* fruit have indicated the presence of various secondary metabolites, that are known to have different therapeutic applications such as anti-hemorrhagic, antimicrobial and antioxidant properties (Supraja and Usha, 2013). In the present study, the antimicrobial efficacy CuNps and crude extracts were analysed using agar well and disc diffusion method. The results were represented as follows:

Disc diffusion method

The antimicrobial activity of the crude and copper mediated nanoparticles of *M. charantia* extract were investigated measuring the zones of inhibition of bacterial and fungal species and were presented in table 1.

The antimicrobial activity against *Staphylococcus aureus* showed that CuNps had the highest zone of inhibition of 20.013 mm while the least zone of inhibition was for ethanol crude extract (5.067 mm) (Fig. 8a) and samples were significantly differed when compared at $p < 0.05$.

Similarly, the antimicrobial activity against *E. coli* was also screened (Fig. 8b). The result showed that the zone of inhibition was in the order; ampicillin > CuNps > crude extracts (i.e. 14.03mm > 11.07 mm > 2.83mm). On the other hand, CuNps and crude extracts didn't have significant inhibitory activity against *Pseudomonas aeruginosa* (Fig. 8c). The present study also revealed that all the assayed samples showed no activity against the fungi *Aspergillus flavus* (Fig. 8d) and were comparable with studies of Kumar *et al.*, 2010 showing active anti-bactericidal activity

but no activity against fungi or yeast. The comparable antimicrobial of the green synthesized CuNps could imply that ethanol is the suitable media for liberation of phytochemical constituents screened (unpublished data) responsible for antimicrobial activity.

The antimicrobial activity of triterpenes depends on interaction between their lipid components with the net surface charge of microbial membranes. Furthermore, the bioactives might cross the cell membranes, penetrating into the interior of the cell and interact with intracellular sites critical for antibacterial activity (Trombetta *et al.*, 2005). The mechanism by which the nanoparticles were able to penetrate the was due to changes in membrane morphology that significantly increases permeability and affects proper transport through the plasma membrane (Auffan *et al.*, 2009; Brayner *et al.*, 2006), leaving the bacterial cells incapable of properly regulating transport through the plasma membrane and resulting in cell death. It is also said that the nanoparticles penetrates inside the bacteria causing damage to phosphorus and sulfur containing compounds such as DNA (Kirchner *et al.*, 2005).

Table.1 Antimicrobial activity of samples against selected micro organisms using disc diffusion method

S. No	Sample	<i>S. aureus</i> zone of inhibition (mm)	<i>P. aeruginosa</i> zone of inhibition (mm)	<i>E. coli</i> zone of inhibition (mm)	<i>A. niger</i> zone of inhibition (mm)
1	Crude ethanol extract	5.07 ± 0.02 ^b	2.07 ± 0.05 ^c	2.83 ± 0.03 ^c	-
2	CuNps	20.013 ± 0.02 ^a	9.51 ± 0.02 ^b	11.07 ± 0.04 ^b	-
3	Ampicillin	4.97 ± 0.06 ^c	19.80 ± 0.2 ^a	14.03 ± 0.04 ^a	-

Key – No Inhibition

Note: Values are expressed as mean ± standard deviation of triplicates. Mean values with similar superscripts within a column row do not difference significantly

Table.2 Antimicrobial activity of samples against selected microorganisms using agar well diffusion

S. No.	Sample	<i>S. aureus</i> Zone of Inhibition (mm)	<i>P. aeruginosa</i> Zone of Inhibition (mm)	<i>E. Coli</i> Zone of Inhibition (mm)	<i>A. niger</i> Zone of Inhibition (mm)
1	Crude extract	0.33 ± 0.046 ^e	2.01 ± 0.017 ^e	-	-
2	CuNps	10.04 ± 0.036 ^a	10.53 ± 0.044 ^a	-	-
3	Ampicillin	7.057 ± 0.051 ^b	8.17 ± 0.05 ^b	-	-

Key – No Inhibition

Note: Values are expressed as mean ± standard deviation of triplicates. Mean values with similar superscripts within a column row do not difference significantly.

Figure.1 Observed colour change after 24hrs – Formation of CuNps



Figure.2 U.V Visible spectrum of CuNps synthesized from *M. charantia*

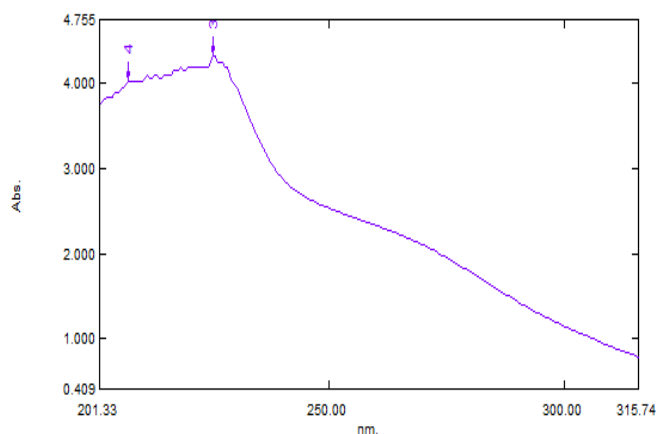


Figure.3 DLS measurement for CuNps synthesized from ethanol extract of *M.charantia*

Calculation Results

Peak No.	S.P. Area Ratio	Mean	S. D.	Mode
1	1.00	98.4 nm	25.2 nm	88.0 nm
2	--	-- nm	-- nm	-- nm
3	--	-- nm	-- nm	-- nm
Total	1.00	98.4 nm	25.2 nm	88.0 nm

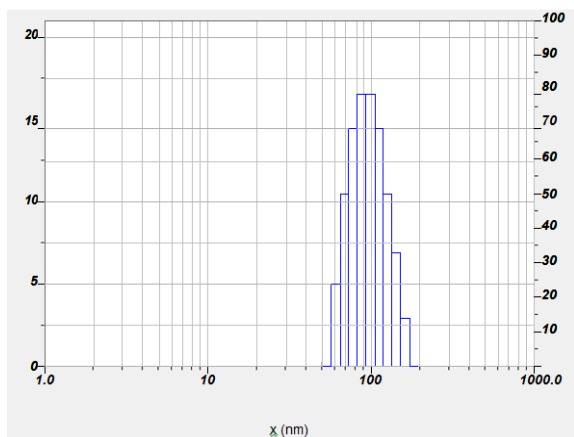


Figure.4 Zeta potential measurement for CuNps synthesized from ethanol extract of *M.charantia*

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	8.4 mV	0.000066 cm ² /Vs
2	81.0 mV	0.000628 cm ² /Vs
3	127.2 mV	0.000987 cm ² /Vs

Zeta Potential (Mean) : 30.0 mV

Electrophoretic Mobility mean : 0.000233 cm²/Vs

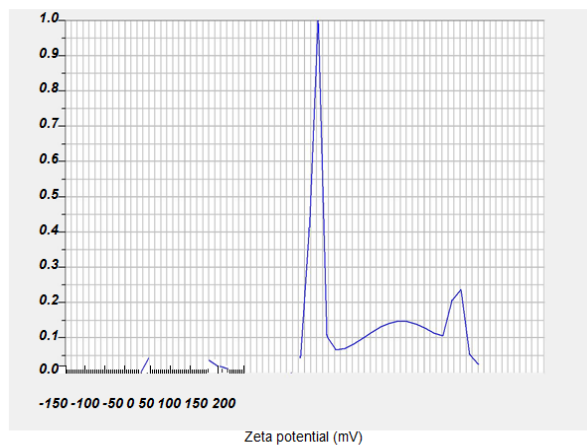


Figure.5 TEM image of CuNps synthesized from *M. charantia*

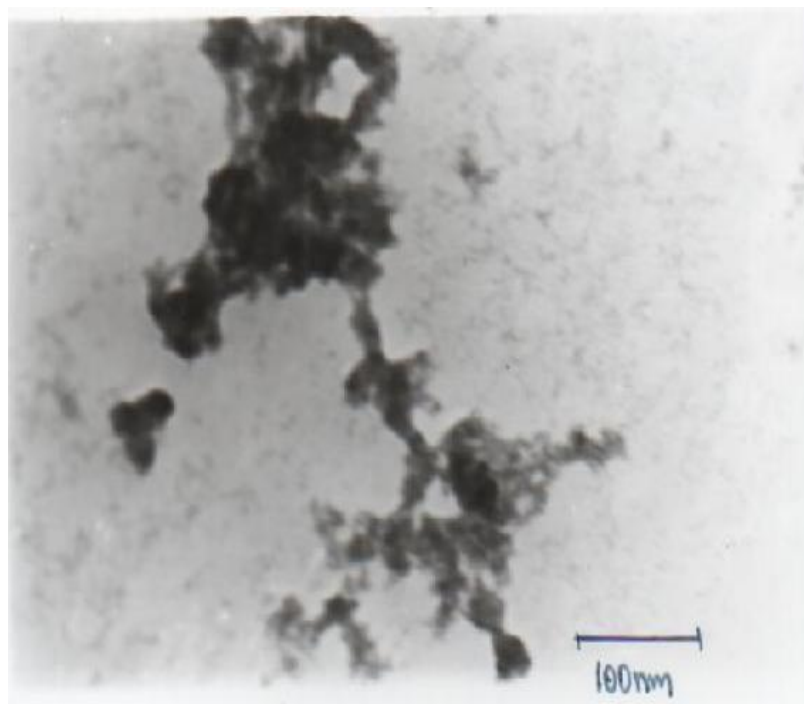


Figure.6 FTIR spectrum recorded from CuNps of *M. charantia* extract

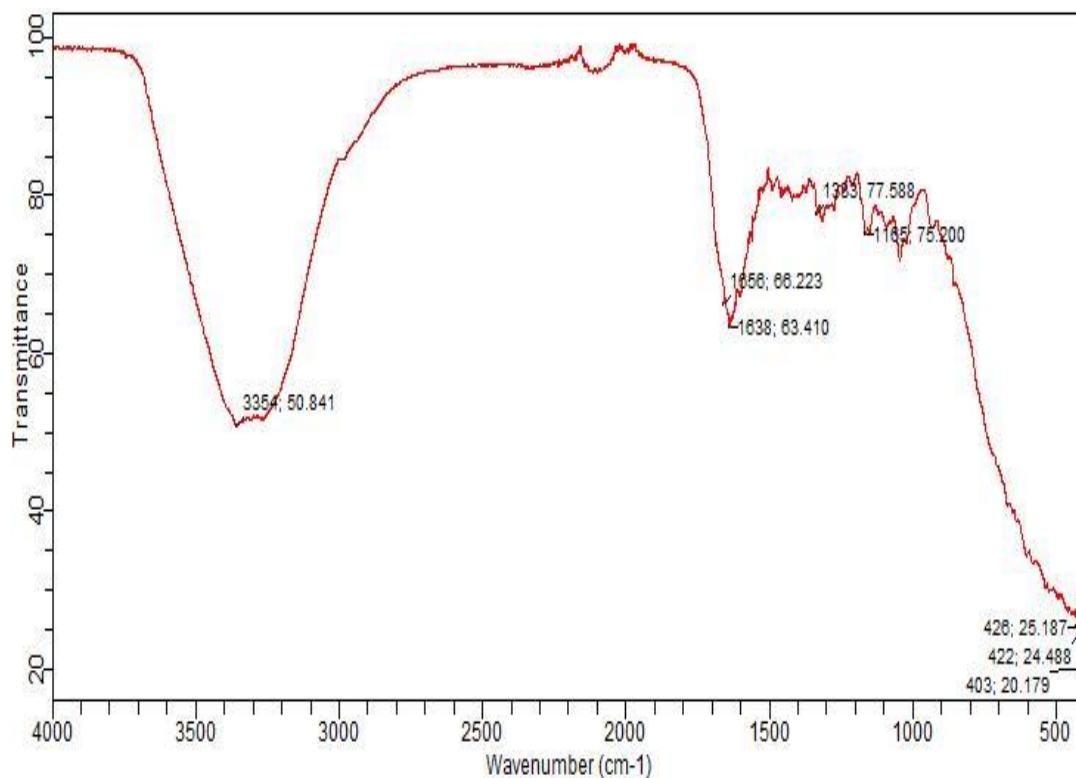


Figure.7 XRD micrograph of CuNps synthesized using ethanol extract of *M. charantia*

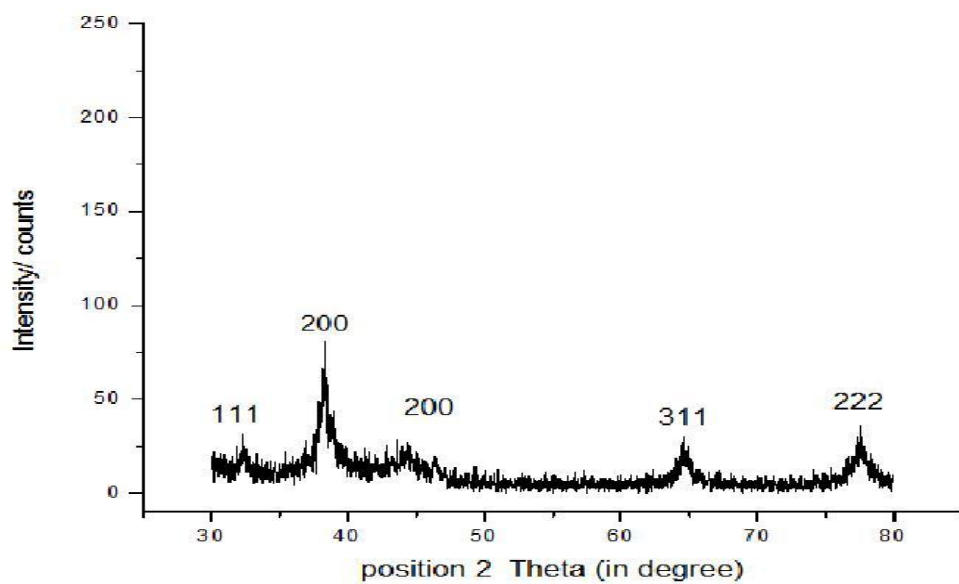


Figure.8 Antimicrobial activity of *M. charantia* against different micro organisms

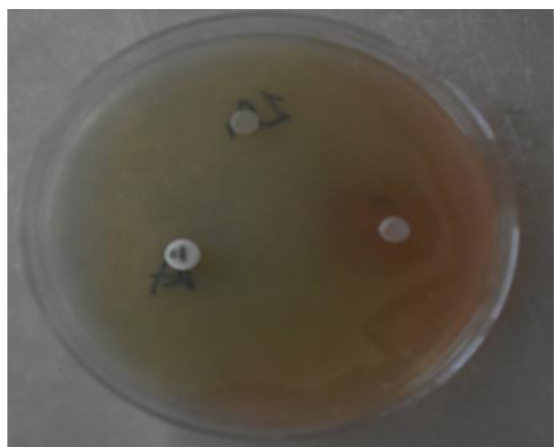


Figure.8a *S. aureus*

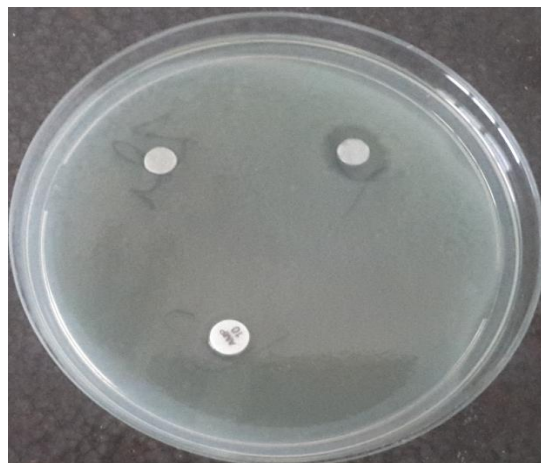


Figure.8b *P. aeruginosa*

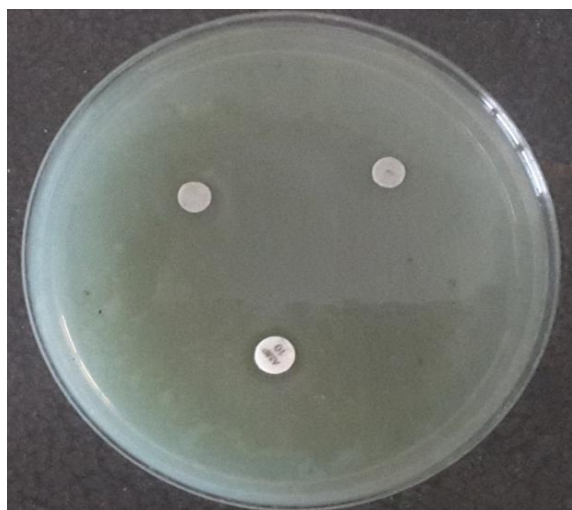


Figure.8c *E. coli*



Figure.8d *A. flavus*

Various researchers have also shown that gram positive bacteria are more susceptible to plant extracts than gram negative bacteria. The cell wall in gram positive bacteria is of a single layer whereas the gram negative cell wall is multi-layered (Parekh and Chanda, 2007).

The other possible mechanism of action, responsible for the enhanced activity of mediated CuNPs could be the improved characteristics and morphological properties of nanoscale materials in terms of specificity and better manipulation, increased surface area available for interactions, which

enhances bactericidal effect than the large sized particles and thus, they impart cytotoxicity to the microorganisms (Adams *et al.*, 2006; Supraja *et al.*, 2015).

Agar well diffusion method

The ethanol extract of *M. charantia* and its derivative CuNPs were evaluated for their broad spectrum of activity on selected bacterial and fungal strains using agar well diffusion assay.

Staphylococcus aureus and *Pseudomonas aeruginosa* were sensitive to all the assayed

samples while *E. coli* and *Aspergillus flavus* showed no sensitivity at all (Table 2). This justifies the traditional use of the plant against *S. aureus* with clinical significance against variety of suppurative (pus forming) infections and toxinoses in humans. Not in exception was *P. aeruginosa* which caused a good number of infections such as septic burns and wounds, conjunctivitis, endocarditis, meningitis and urinary tract infections (Michael *et al.*, 1999).

Comparison of inhibition zones of the samples against *S. aureus* shows that the assayed crude extract was not as potent as the derivative CuNps and the standard ampicillin. Maximum inhibition was observed for CuNps (10.057 mm) which differed significantly from ampicillin (7.057 mm) at $p < 0.005$.

It has been reported that higher plants like wise show a promising potential source of new anti-microbial agents due to certain phytochemicals (Selvamohan *et al.*, 2012). In spite of this, it would not be unusual for *S. aureus*, *E. coli* and *P. aeruginosa* if they were resistant to the assayed extracts because of their multidrug resistance characteristics. The consistent observed resistance of *A. flavus* in both methods used in this study to evaluate the anti-microbial activity of *M. charantia* and its derivative nanoparticles might be attributable to the presence of more active enzymes in these microbes which deactivate the active antimicrobial components due to low affinity of the active component(s) on the target molecules. In line to our findings, other previous studies have demonstrated both *in-vitro* and *in-vivo* antibacterial activities against *E. coli*, *Staphylococcus*, *Pseudomonas*, *Salmonella*, *Streptobacillus* and *Streptococcus* by the plant extracts of bitter gourd (Ozusaglam and Karakoca, 2013).

Furthermore, copper which have been used as an anti-microbial agent for decades has

revealed a strong antibacterial activity and showed good potency in this study. Copper oxide (CuO) nanoparticles have been reported to act as potential antimicrobial agent against infectious organisms such as *E. coli*, *Bacillus subtilis*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Syphillis typhus*, and *Staphylococcus aureus* (Shobha *et al.*, 2014).

In conclusion, although the use of antibiotics has greatly reduced the incidence of infectious diseases, their extensive uses in therapy or as growth promoters in animal food has led to the appearance of drug-resistant bacteria (Normanno *et al.*, 2007), which is a major public health issue worldwide. In order to inhibit food-borne pathogens and to extend shelf life, synthetic chemicals are often used as preservatives in food processing and storage. Consumer awareness over the potential risks of synthetic food additives to human health has renewed the interest in using naturally occurring alternatives. Hence *M. charantia* extracts and nanoparticles screened for antimicrobial properties have potentials in protecting consumers from microbial infection and potential applications in food systems (Serra *et al.*, 2008; Lou *et al.*, 2010). The findings in this study may lead to the development of CuNps-based new antimicrobial systems for eco-friendly applications in packaging, preservation and storage of food as well as bactericidal, wound healing and other medical and electronic applications thus making it potentially exciting for industries.

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