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Original Article

Green synthesis of Copper Nanoparticles using *Curcuma longa* L. and *Azadirachta indica* A.Juss. and their Antibacterial Activity

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ABSTRACT

The bacterial diseases are very emerging one and now a day's most of the drugs have a resistant mechanism. In order to find suitable drugs from the plant based nanomaterials is excellent and effective tool for treat many drug resistant bacteria. In this study, various solvents such as chloroform, petroleum ether, ethanol, ethyl acetate, methanol and n-butanol used to prepare the crude extracts with rhizomes of *Curcuma longa* L. and leaves of *Azadirachta indica* A.Juss. separately. Then these extracts with nanoparticles prepared by using copper acetate monohydrate and copper sulphate pentahydrate solutions. Finally, the sequence of antibacterial activities was done by using of the selected medicinal plant such as rhizome of turmeric and leaves of Neem extracts against gram negative and gram positive bacterial pathogens like *Vibrio cholera* O1 and *Bacillus subtilis* ATCC 6051 respectively. The green synthesis of copper nanoparticles along with plant extracts was collected and the shape and size of copper nanoparticls of the plant extracts were determined in Scanning Electron Microscope (SEM) and Energy Dispersive X-ray Spectroscopy (EDX). The nutrient agar well diffusion method is used to find the antibacterial activities against various plant extracts along with different antibiotic sensitivity tests. The antibacterial activities of the medicinal plant rhizome and leaves of crude extracts were applied in different concentrations in nutrient agar well plates to indicate the presence zone of clearance. The crude extracts were prepared from different solvents such as the ethanol and methanol extracts with copper nanoparticles separately, it was the most effective extracts. At this stage the gram negative bacteria *Vibrio cholerae* and the gram positive bacteria *B. subtilis* appear to be most sensitive strains. The inhibition of microbial growth at concentration as low as ~50 to 150 mg/mL indicated the potent antibacterial activity of above mentioned selected medicinal plant extract copper nanoparticles. In this research works better results were find critically with industrially important compounds from selected plant nanoparticles with their plant compounds is responsible for very excellent antibacterial activity.

INTRODUCTION

The best one of the scientific advancement of the society is nanotechnology is very important tool investigations of medicine, energy, manufacturing, consumer products materials, etc. Nanotechnology is the use of matter on atomic structure or molecular or supra-molecular for industrial purposes have many properties [1]. The nanomaterials play a central role on nanotechnology in physics, chemistry and biology [2]. Recently the biological nanomaterials are very effective tool for the drug carrier in medicine and pharmaceutical industries, etc. Most of the nanomaterials are chemical based and can be classified in two major categories are inorganic and organic nanomaterials. The inorganic nanomaterials include silica, elementary substances, quantum dots and alloys. On the other hand, the organic nanomaterials such as nanofibers, liposomes,

nanotubes, and other polymer substances etc [3]. But these all the synthetic and chemically unsafe in the form of raw use to human use and it can create some side effects. In this situation may change by the application of medicinal plant based crude drugs using with some nanomaterials like chemical elements, copper, silver, cobalt, etc [4,5].

All type of pathogens is called biotic factor which affects the other living organisms especially plants and animal's life cycle. The pathogenic organisms include bacteria, viruses, some protozoan and fungi. The pathogens are predominantly increased in our environment by pollution activities of humans. Currently the bacterial pathogens show the serious effects to humans in particularly drug resistant pathogens. In order to find suitable natural drug is very important. Bacterial pathogens existing in everywhere classified in two main categories, these are gram positive and gram negative and causes deadly diseases to both higher plants and animals [6]. *V. cholerae* O1 type is a species of Gram-negative, facultative anaerobe and comma-shaped bacteria. Cholera can be pandemic, endemic or epidemic. The infectious disease cholera is brought on by these bacteria. The *V. cholerae* present in places with low sanitation and little access to clean drinking water and causes cholera and is mostly spread through the consumption of tainted food or water, itcolonizes the small intestine after being consumed in tainted water or food, where they release cholera toxin [7]. Cholera frequently causes outbreaks in poor nations and also thrives in aquatic habitats like coastal areas and estuaries. The *Bacillus subtilis* ATCC 6051 is a gram-positive and rod-shaped bacterium called *B. subtilis*. It causes serious infectious diseases like sepsis, pneumonia, and wound infections are just a few of the different clinical manifestations that these infections can take. Such incidents are rare, though, and *B. subtilis* is not regarded as a serious human disease, its potential uses in numerous fields have been researched in recent years.

The terrestrial tropical eudicot plants secrete very excellent medicinal secondary metabolites for weapon to fight against abiotic and biotic stresses [8]. The global level higher plants occupy 31% and marine organisms also provide some important medicinal compounds. The higher plants are the major bioresource for many human research and drug discovery activities. Medicinal plants are very important source to treat various kinds of diseases in humans and animals. Among various medicines providing plants, the *Curcuma longa* (L.) is a flowering plant of the ginger family, Zingiberaceae. It is commonly known as Turmeric. The aroma of yellow color of *C. longa* (L.) is due to curcumin. It was isolated from turmeric by solvent extraction. Curcumin is also known as diferuloyl methane, a phytochemical found in the Turmeric. *C. longa* (L.) is also helps to treat in anticancer, antioxidant, anti-inflammatory and antibacterial agents in the ayurvedic or medicinal field has been developed [9]. The efficiency of curcumin act is reducing agent for the synthesis of copper nanoparticles [10]. *A. indica* (L.) is commonly known as neem. It belongs to the family Meliaceae. The neem contains many phytochemicals such as azadirachtin, polyphenols, triterpenes, etc. The leaf contains carotenes, vitamins. It also contains alkaloids, tannins, saponins and minerals that act as a stabilizing agent for metal nanoparticles. *A. indica* (L.) is found in worldwide and chiefly available for even very poor people. It is one of the very best nanomaterials to cure variety of human diseases like both infectious ad non-infectious diseases [11]. Most indians and other countries families recommended neem leaves as hindu goddess pooja material to remove the body abscess, acne wounds, antihelminthic and excellent antiseptic agent and the young stem of neem plant is used for treating the tooth decay and it gum infections.

All land plants considered as medicinal plants and are the alternative source used to develop new, novel drugs from its secondary metabolites. Previously the medicinal plants with their nanoparticles were used to done their antibacterial activity and ultimately not investigated predominantly. In this study to find the antibacterial activity of selected bacterial pathogens namely *V. cholerae* O1 and *B. subtilis* ATCC 6051in selected medicinal plants which includes rhizome of *C. longa* (L.) & leaves of *A. indica* (L.) against various solvents like ethanol and methanol. The green synthesized nanoparticles are very eminently created the zone of clearance against selected pathogens in disc or agar well diffusion petriplates. The nanoparticles size and quantity were analyzed by scanning electron microscope and Energy Dispersive X-Ray Analysiswith UV visible spectroscopy techniques. These techniques employed to find the selected medicinal plants nanoparticles with secondary metabolites is responsible to remove or intact the bacterial metabolites, nucleic acids or

proteins in the antibacterial activity zone of clearance.

MATERIALS AND METHODS

Plant Collection

C. longa (L.) and *A. indica* (L.) were collected from Vivekanandha College of Arts and Sciences and Tirunagarcology, Tiruchengoderegions of Namakkal District, Tamil Nadu. The taxonomical identification of these medicinal plants was done by Department of Botany, Vivekanandha College of Arts and Sciences, Tiruchengode.

Plant Preparation and Extraction

The fresh dry rhizome of *C. longa* (L.) and fresh leaves from *A. indica* (L.) was washed well under running tap water and dried in a warm place for 3 to 5 days. The samples were grinded into fine powder and extract prepared by different solvents such as ethanol, methanol with green synthesis of copper nanoparticles for antibacterial activity analysis.

Extraction of *C. longa* (L.)

10 g of *C. longa* (L.) powder was dissolved in 100 ml of ethanol in 200ml conical flask. This extract was kept in waterbath at 70 ℃ for 2 hours. It was filtered by using aluminium foil.

Extraction of *A. indica* (L.)

10 g of *A. indica* (L.) leaf extract was dissolved in 100 ml of methanol in 200 ml conical flask. This extract was kept in waterbath at 70 ℃ for 2 hours. It was filtered by using aluminium foil.

Synthesis of Copper Nanoparticles in *C. longa* (L.) Extract

Copper acetate monohydrate solution (0.1ml/100ml) was taken and 50 ml of *C. longa* (L.) extract was added to it. The color changed from yellow to brown color indicates the presence of copper nanoparticles [12] was analyzed in UV- Visible Spectroscopy.

Synthesis of Copper Nanoparticles in *A. indica* (L.) Extract

Copper sulphate pentahydrate solution (0.1ml/100ml) was taken and 50 ml of *A. indica* (L.) extract was added to it. The color changed from pale green to dark green color indicates the presence of copper nanoparticles [12] was also analyzed in UV-Visible Spectroscopy.

Bacterial Culture Media Preparation and Inoculation

The bacterial pathogenic microorganisms are used for the test were *V. cholera* O1and *Bacillus subtilis* ATCC 6051. The nutrient agar was used as the media for culturing of strains. Loops full of the microbial cultures were inoculated in the nutrient broth at 37 ℃ for 72 hours. Both bacterial cultures were streaked and maintained in Luria Bertani agar plates. For Antibacterial Assay, both the strains were cultivated in sterile 2 ml of Nutrient broth for overnight and 1% inoculums was sub-cultured for 3 hours in 2ml of sterile Nutrient broth or Luria Bertani broth.

Antibacterial Activity

The agar well diffusion method was performed in nutrient agar plates. The nutrient agar medium was poured into sterile petriplates. The plates were allowed to solidify. Then the sub cultured test pathogens such as Gram negative bacteria *V. cholera* and Gram positive bacteria *B. subtilis* were swabbed on each nutrient agar plates. Chloramphenicol antibiotics discs was used as a positive control with the help of 1ml tips and wells were developed and at different concentrations such as 20µl, 40µl, 60µl and 80µl. The samples were loaded and spread using L-rod on each nutrient agar plates. The zone of inhibition was observed against each pathogen [13].

Characterization of Copper Nanoparticles in *C. longa* (L.) and *A. indica* (L.)

The synthesized copper nanoparticles of *C. longa* (L.) and *A. indica* (L.) were analyzed by UV-Visible Spectroscopy. The spectrum analysis of copper nanoparticles (CuNPs) was scanned from 300 to 800 nm and the absorbance value was noted. The production of copper nanoparticles of *C. longa* (L.) and *A. indica* (L.) were centrifuged at 2000 rpm for 10 minutes. The supernatant was removed and the pellet was collected. The pellet was kept at hot air oven for overnight at 37 ℃. It was air dried and analysed for scanning electron microscope (SEM) and energy dispersive x-ray spectroscopy (EDAX). The morphological characteristics of copper nanoparticles were studied by scanning electron microscope (SEM). The 1-cm-diameter aluminium stub was used on the sample holder and cleaned with acetone and compressed air to remove any surface oils or dirt. The double-coated conductive carbon

tape was applied to the stub with adhesive. A thin coating of the dried sample approximately 0.2ml was deposited on the adhesive surface, and it was then coated with palladium using a sputter coater for around 90s to make the samples conductive. To see clearly the shape of copper nanoparticles at an accelerating voltage of 1 to 20 kv at working distance of the sample holder was removed from the sputter coater and inserted in the vacuum chamber of the Scanning Electron Microscope (SEM) and a 1 to 30,000 times magnification was used to see clearly the shape of copper nanoparticles. The distance of operation of the sample is 10mm and the acceleration voltage varies between 1 to 20 kv and thin layer of dried sample at the acceleration of 30 kv analyzed using EDAX of copper nanoparticles plant extracts.

RESULTS

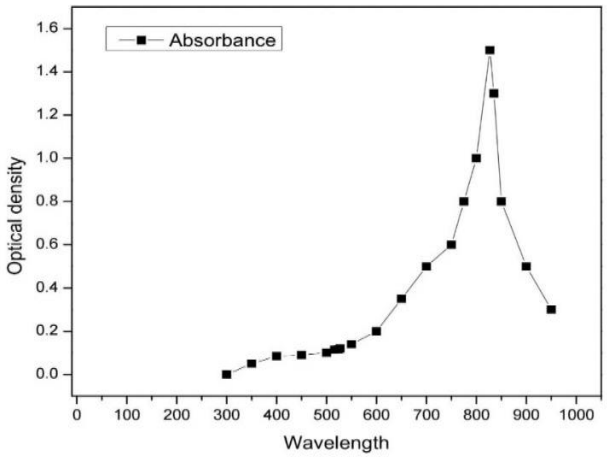

Fig. 1 The UV-Visible Spectroscopy analysis of CuNPs in *C. longa* (L.)

Characterization of Copper Nanoparticles

Preparation of Plant Extracts

The dried samples powders of *C. longa* (L.) and *A. indica* (L.) were extracted with solvents like ethanol and methanol.

Synthesis of Copper Nanoparticles

The *C. longa* (L.) extracts turns into brown colour which indicates the copper nanoparticles (CuNPs) were synthesized and the *A. indica* (L.) extracts turns into dark green colour which indicates the copper nanoparticles (CuNPs) were synthesized.

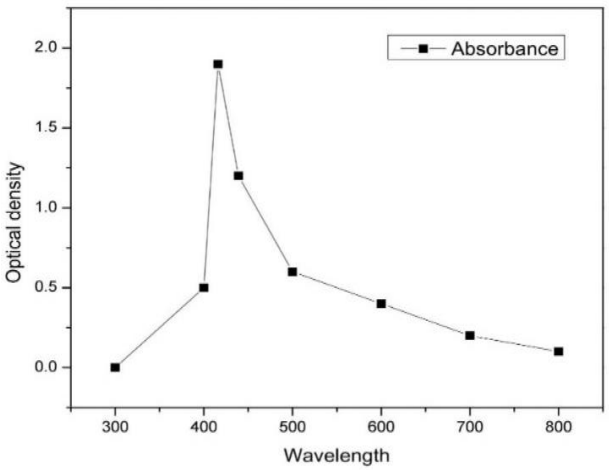

Fig. 2 The UV-Visible Spectroscopy analysis of CuNPsin *A. indica* (L.)

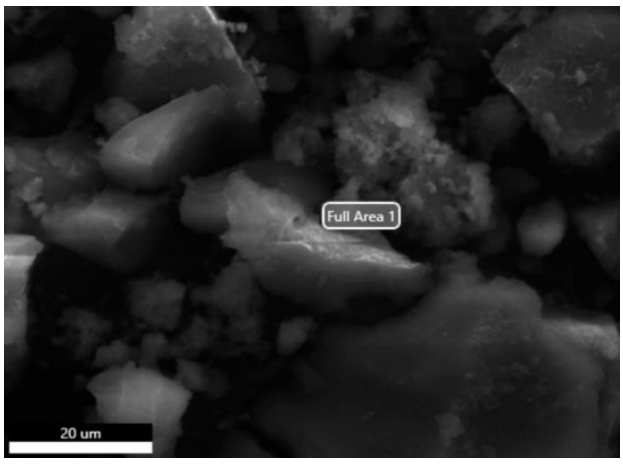

Fig. 3 SEM analysis of copper nanoparticles in *C. longa* (L.)

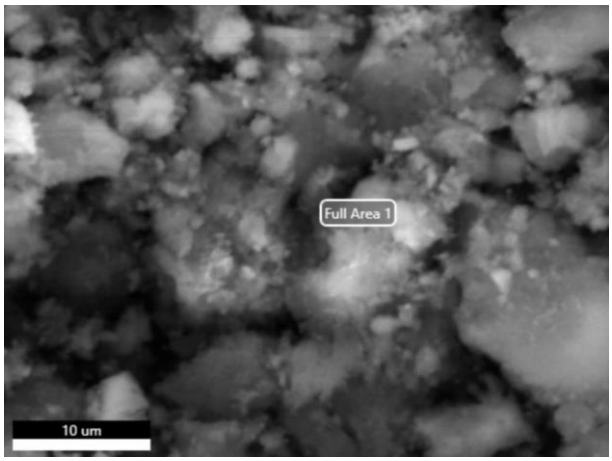

Fig. 4 SEM analysis of copper nanoparticles in *A. indica* (L.)

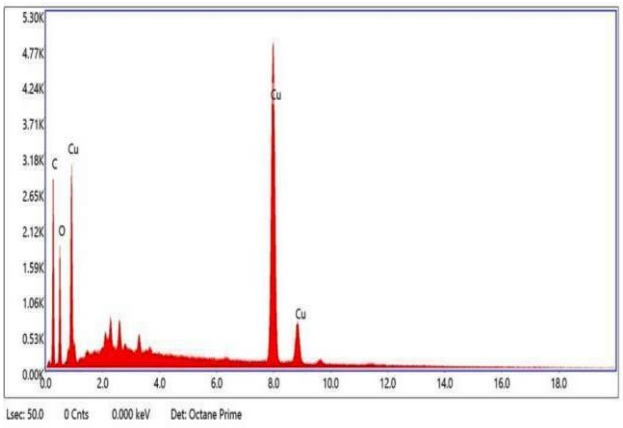

Fig. 5 EDAX analysis for the presence copper nanoparticles in the *C. longa* (L.)

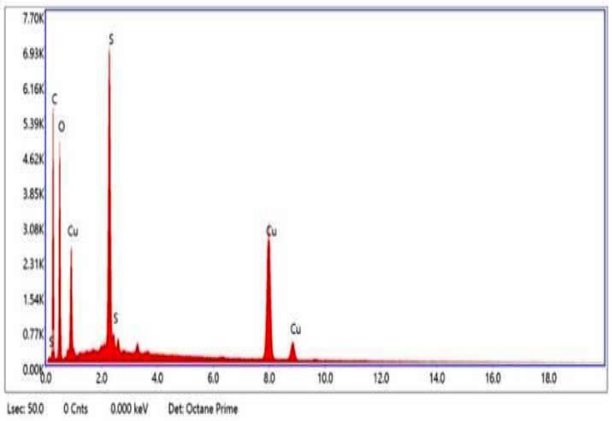

Fig. 6 EDAX analysis for the presence copper nanoparticles in the *A. indica* (L.)

UV-Visible Spectrophotometer Analysis

The synthesized copper nanoparticles were analysed by using UV-Visible Spectroscopy. The wavelength and absorbance value was observed and it is plot in graph. The graph is given below in figure 1 and 2. At 516 nm, the absorbance of copper nanoparticles is 0.116. At 527nm, the absorbance value is reduced to 0.115.

The highest peak value is at 827 nm and its absorbance value is 1.5 were observed in UV-Visible spectrophotometer. At 439 nm, the absorbance value is 1.474. The highest peak value observed at 416 nm and its absorbance is 1.973 were observed in the UV-Visible spectrophotometer.

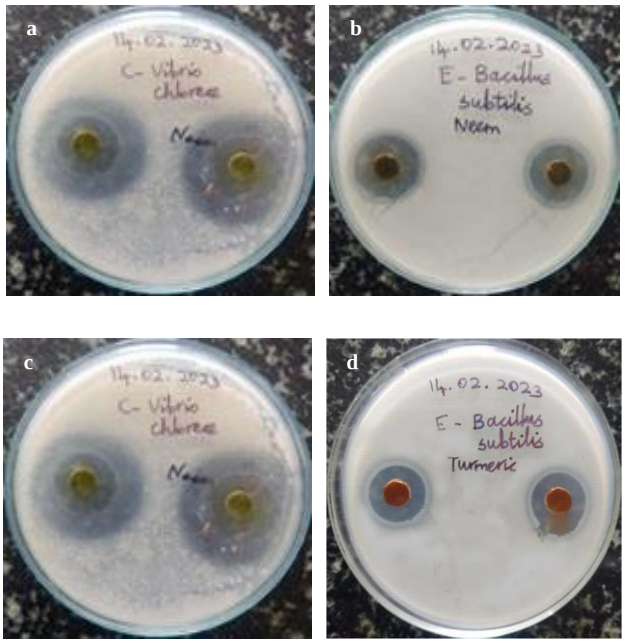

Fig 7: 1 The figure 7(a & b) and figure 7 (c & d) shows that the zone of inhibition of synthesized copper nanoparticles in *C. longa* (L.) of ethanol and *A. indica* (L.) of methanol against *V. cholerae* and *Bacillus subtilis*.

Scanning Electron Microscope (SEM) Analysis

The SEM analysis were determined the structure of copper nanoparticles present in the sample.

Table 1 EDAX analysis for the presence copper nanoparticles in the *C. longa* (L.)

Elements	Weight%	Atomic%	Net Int	Error%	Kratio	Z	Z	F
C K	49.62	66.60	317.27	8.24	0.1614	1.0812	0.3296	1.0000
O K	22.03	24.32	204.01	10.45	0.0341	1.0448	0.1483	1.0000
S K	3.47	3.13	516.37	2.41	0.0316	0.9234	0.8862	1.0051
CuK	32.67	9.08	1521.05	1.74	0.2758	0.8076	1.0290	1.0162

Table 2 EDAX analysis for the presence copper nanoparticles in the *A. indica* (L.)

Elements	Weight%	Atomic%	Net Int	Error%	Kratio	Z	Z	F
C K	49.62	62.43	629.17	8.16	0.1603	1.0406	0.3105	1.0000
O K	34.32	32.42	561.22	10.14	0.0470	1.0033	0.1366	1.0000
S K	5.70	2.69	1328.34	2.94	0.0435	0.9095	0.8330	1.0069
CuK	10.36	2.46	961.22	2.32	0.0873	0.7679	1.0339	1.0617

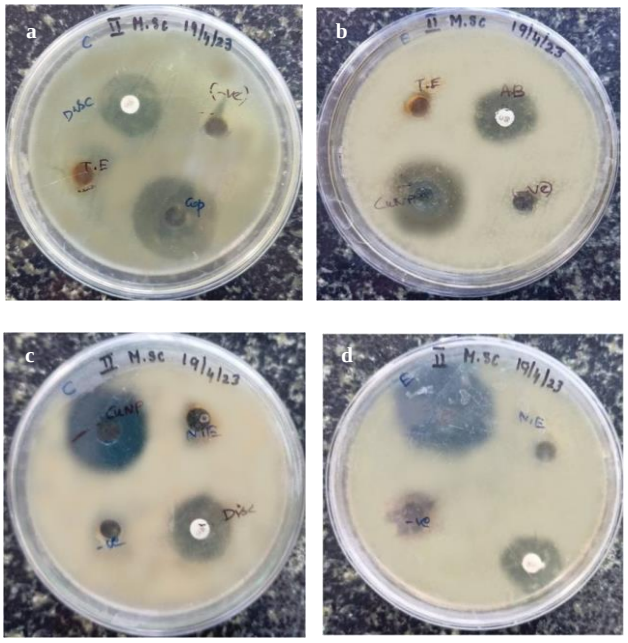

Fig 8: 2 The figure 8 (a & b) and 8 (c & d) shows that the zone of inhibition antibacterial activity of control chloramphenicol and copper nanoparticles of *C. longa* (L.) and *A. indica* (L.) of different solvents against *V. cholerae* and *Bacillus subtilis*.

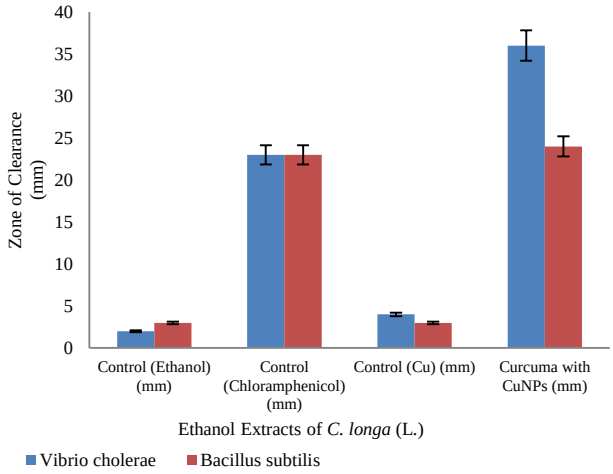

Fig. 9 Antibacterial activity of Control vs CuNPs in *C. longa* (L.) extract.

The highest zone of inhibition occurs in *V. chloreae* of *C. longa* (L.) extract of copper nanoparticles

Energy Dispersive X-Ray Analysis (EDAX) Method

The EDAX analysis is indication of the presence of copper nanoparticles in synthesized samples.

Antibacterial Activity

The antibacterial activity analysed for the copper nanoparticles incorporated plant extract of dried samples of *C. longa* (L.) and *A. indica* (L.) were extracted with solvent systems like ethanol and methanol against two test pathogenic samples like *V. cholera* O1and Bacillus subtilis ATCC 6051 [13].

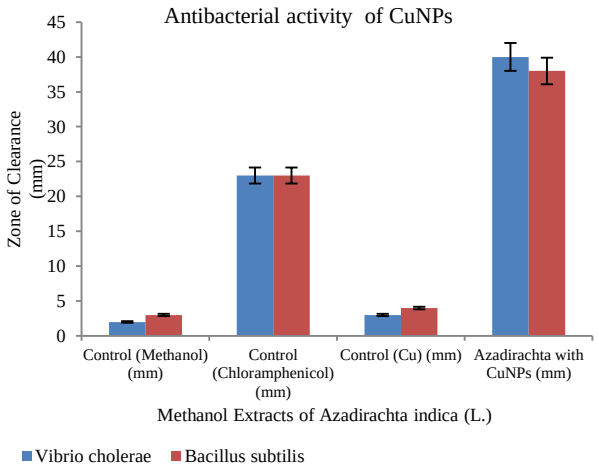

Fig. 10 Antibacterial activity of Control vs CuNPs in *A. indica* (L.) extract.

The highest zone of inhibition occurs in *V. chloreae* of *C. longa* (L.) extract of Copper Nanoparticles.

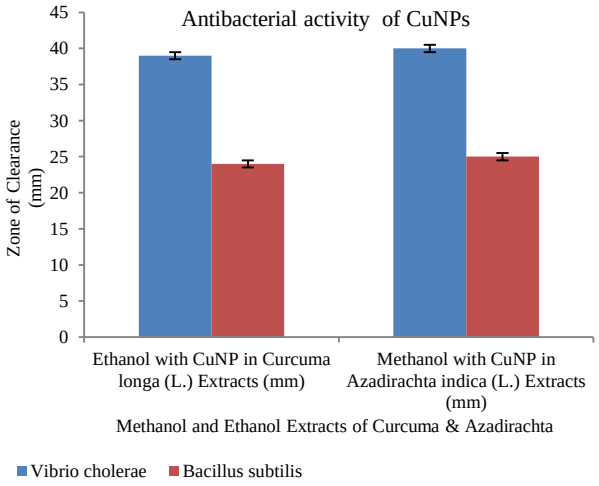

Fig. 11 Antibacterial activity of copper nanoparticles in *C. longa* (L.) and *A. indica* (L.) against *V. chloreae* and *B. subtilis*

The highest zone of inhibition occurs in *V. chloreae* of *C. longa* (L.) extract of copper nanoparticle

STATISTICAL ANALYSIS

In this experiment the two bacterial pathogens used to analysis the antibacterial activity has been carried out in Table 4 and 5 have many statistical assessment tests by means of measures of positions like Mean, Median, and Mode [14,15].

Table 3 Antibacterial activity of Control vs CuNPs in *C. longa* (L.)

Pathogens	Control (Ethanol) (mm)	Control (Chloramphenicol) (mm)	Control (Copper) (mm)	*Curcuma* + Ethanol extracts with CuNPs (mm)
V. cholerae	02	23	04	36
B. subtilis	03	23	03	24

Table 4 Antibacterial activity of Control vs CuNPs in *A. indica* (L.)

Pathogens	Control (Methanol) (mm)	Control (Chloramphenicol) (mm)	Control (Cu) (mm)	*Azadirachta* + Methanol extracts with CuNPs (mm)
V. cholerae	02	23	03	40
B. subtilis	03	23	04	38

Table 5 Antibacterial activity of Copper Nanoparticles in *C. longa* (L.) and *A. indica* (L.) in Ethanol and Methanol extracts

Bacterial species	Mean	Median	Mode	Standard Deviation	Standard Error	T-Test	Probability
V. cholerae	39	39	39	1.1258	0.4742	58.483	$P < 0.001$
B. subtilis	24	24	25	0.7411	0.2217	43.483	$P < 0.001$

Table 6 Statistical Analysis of zones of clearance size (mm) in *C. longa* (L.) with CuNPs ethanol extracts

Bacterial species	Mean	Median	Mode	Standard Deviation	Standard Error	T-Test	Probability
V. cholerae	40	40	40	1.1163	0.5042	59.537	$P < 0.001$
B. subtilis	25	25	25	0.8214	0.2617	46.483	$P < 0.001$

Table 7 Statistical Analysis of zones of clearance size (mm) in *A. indica* (L.) with CuNPs methanol extracts

Pathogens	Ethanol with CuNP in *C. longa* (L.) Extracts (mm)	Methanol with CuNP in *A. indica* (L.)Extracts (mm)
V. cholerae	39	40
B. subtilis	24	25

These tests are used to find the effectiveness of extracts against each bacterium in the form of different size of zone of clearance in millimeter (mm). The standard error, T value, Probability and Standard deviation were calculated effectively and obtained different significant values on the form of better outcomes.

In table 6 illustrate the prominent antibacterial activity of *C. longa* (L.) against *V. cholerae* and *B. subtilis*. The *V. cholerae* was shows the high sensitive to this turmeric CuNPs, it means that higher zone of clearance whereas the *B. subtilis* the least susceptibility to these extracts. The extract of *C. longa* (L.) showed significant antibacterial activity against bacterial pathogens although it was in the form of CuNPs. The standard deviation and T-test values show very high significant results and as the probability are less than 0.001 in all cases of bacterial pathogens.

In table 7 illustrate the prominent antibacterial activity of *A. indica* (L.) against *V. cholerae* and *Bacillus subtilis*. The *V. cholerae* was shows the high sensitive to this turmeric CuNPs, it means that higher zone of clearance whereas the *B. subtilis* the least susceptibility to these extracts. The extract of *A. indica* (L.) showed significant antibacterial activity against bacterial pathogens although it was in the form of CuNPs. The standard deviation and T-test values show very high significant results and as the probability are less than 0.001 in all cases of bacterial pathogens.

DISCUSSION

The creation of nanoparticles uses a variety of physical and chemical techniques. Strong and weak chemical reducing agents as well as protecting agent like zinc, sodium borohydride, silver, copper, sodium citrate and alcohols are needed to use these synthesis techniques [16]. It prompts to the quest for alternatives that might be environmentally friendly methods.

Similarly, in this work is based on copper nanoparticles produced from copper acetate monohydrate and copper sulphate pentahydrates. *Curcma longa* (L.) extract was mixed with copper acetate monohydrate used creation of better nanoparticles for drug targeting purposes [17] similar results of our study reporting that the *C. longa* (L.) extract was mixed with copper acetate monohydrate and copper sulphate pentahydrates before and after microwave irradiation and the colour of the solution gradually turned to dark brown which indicated the reduction of copper nanoparticles. The UV-Visible spectroscopy absorption peak for nanoparticles identification [18] in the same way here the UV-Visible spectroscopy absorption peak was observed at 516 nm and may be due to the surface plasma on band of copper colloids formation of non-oxidized copper nanoparticles and is the confirmation of production of copper nanoparticles (Fig. 1).The SEM is the key for identification of copper nanoparticles creation and identification [19], similarly in the present study, the scanning electron microscope (SEM) image of copper nanoparticles were analysed and the size is in 10 to 20µm (Fig. 3). The EDAX analysis of certain plant extract confirmed the presence of copper Nanoparticles [20], same likely that the Energy Dispersive X-Ray Analysis (EDAX) analysis of *C. longa* (L.) extract confirmed the presence of copper nanoparticles (Fig. 5). The antibacterial activity of copper nanoparticles of *C. longa* (L.) extract shows the highest zone of inhibition (Fig. 7a-b, Fig. 8a-b, Table 3, Fig. 9, Table 5 and Fig. 11) against bacterial pathogens such as *V. chloreae* and *Bacillus subtilis* when compared to the same like result of antibacterial activity were observed [21].Some scientific reports showed that, most medicinal plants like *A. indica* (L.) of methanol extract was mixed with copper sulphate pentahydrate, the color of the solution gradually turned to pale green which indicates the reduction of copper nanoparticles [22], in these studies same results were observed in *A. indica* (L.). The UV-Visible Spectroscopy described that the *A. indica* (L.) mediated copper nanoparticles [23], in the same path that like in this research study was observed at 416 nm (Fig. 2).

The SEM image of synthesized Nanoparticles was found to be 20 µm with same results [24] were analysed same like here the analysis of scanning electron microscope (Fig. 4). The EDAX and confirmed the presence of copper nanoparticles [25] similarly in these study results observed in *A. indica* (L.) (Fig. 6). The antibacterial activity of neem plant *A. indica* (L.) extracts showed the zone of inhibition at 10-15 mm [26], similarly we get the 39 mm to 40 mm zone of inhibition observed against *V. cholerae* in the same medicinal plant is the better and highest result in this study (Fig. 7c-d, Fig. 8c-d, Table 4, Fig. 10, Table 5 and Fig. 11). However, in the present study, the antibacterial activity against both*V. Chloreae*and *B. subtilis*showed the highest zone of inhibition like 23 mm, 23 mm, 24 mm, 25 mm, 39 mm, 39 mm and 40 mm were observed in both *C. longa* (L.) and *A. indica* (L.) extracts (Table 5 and Fig. 11). The statistical probability analysis of the results is less than 0.001 in all cases of bacterial pathogens (Table 6 & 7). The green synthesis contributes to raising awareness of the value of plants. Since toxic chemicals are not used in the manufacturing process, using environmentally friendly resources for the synthesis of nanoparticles, such as plant extracts, has many advantages in terms of sustainable development and compatibility for pharmaceutical, cosmetic, and other biomedical applications.

CONCLUSION

In the present study indicates the presence of an active antibacterial compounds in medicinal plants such as *A. indica* (L.) and *C. longa* (L.). The results support that the medicinal plants both *Curcuma* and *Azadirachta* could be a predominant source of novel substances for future drug target to many animal or human diseases like both communicable and non-communicable diseases. A detailed investigation has to be done with the objective of isolating biologically important active compounds nanoparticles along with the search for new very novel macromolecule nanomaterials is currently under the study.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this research paper.

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