ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 7, Issue 2, pp: (202-212), Month: April - June 2019, Available at: www.researchpublish.com

GENERATION AND EVALUATION OF COPPER NANO PARTICLES FROM SELECTED SPECIES OF ANNONACEAE

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Abstract: **Copper bio-nanoparticles are synthesized from the leaves of the selected species of annonaceae by applying green chemistry. The bio-synthesized nanoparticles were characterized by UV-Vis spec. and SEM analysis. Further the copper nanoparticles were screened against both gram positive and negative bacteria. The investigation also looked into the seed germination assay to investigate the toxicity of synthesized nanoparticles. Study reveals that three annonacean members, *Annona squamosa* L., *Annona reticulata* L. and *Annona muricata* L. is capable of reducing copper into copper nanoparticles and the biologically synthesized copper nanoparticles have good bactericidal property and were found to nontoxic by seed germination.**

Keywords: **Copper nanoparticles, Phytochemicals, Antimicrobial activity, SEM analysis, Seed germination assay.**

1. INTRODUCTION

According to the UN, worldwide population will reach 10 billion by 2050[1]. New infectious diseases are continued to evolve by means of changes in human population, behavior, land etc. Infectious diseases remain one among the leading cause of death in less developed nations, especially by bacterial infections. Plants have been a source of medicine to cure various diseases for thousands of years[2]. The use of plant compounds for pharmaceutical purposes has gradually increased worldwide. Phytochemicals are biologically active naturally occurring chemical compounds found in the plants, which provides many health benefits for humans [3]. In the last few years, a number of studies have been conducted in different countries to prove such efficiency[4-7].

Nanotechnology involves the manipulation of matter to create 'nano' sized particles/matter. Nanoparticles are playing a very important role in various fields including medicine. Nanotechnology offers a good opportunity to enhance the efficacy of bio-molecules by decreasing the particle size, thus increasing surface to mass ratio compared to bulk equivalents [8]. Several studies cleared that nanoparticles have the capacity against many diseases including cancer (Breast cancer), tumors, arthritis, HIV-AIDS, influenza etc. There are even reports of their possible use in aqueous nasal sprays for treatment of Alzheimers disease[9].

Currently there are many methods for synthesizing nanoparticles, including chemical precipitation method, thermal decomposition, surfactants, chemical methods, hydro thermal method[10-12]. Recently green nano-technology is involved in the synthesis of bio nanoparticles. Furthermore biomolecules of plants can be used as reducing and capping agents for the synthesis of metal nanoparticles, where by minimizing the use of toxic substances[13].

Family Annonaceae commonly known as soursop family has been long utilized by communities in forest areas where it found[14]. The Annona genus consist of about 119 species most of which are shrubs and trees widely distributed in the tropical and subtropical regions, it have been used as vermifuges, anti-inflammatory agents and in the treatment of

diarrohea and dysentry. All these properties are due to the presence of various phytochemicals present in the plants[15]. Present study investigated the antimicrobial activity of copper nanoparticles synthesized from the leaf extracts of *Annona squamosa* L., *Annona muricata* L. and *Annona reticulata* L (Fig – 1). and also to check the toxicity of the synthesized nanoparticles by seed germination assay.

FIGURE 1: PLANTS UNDER INVESTIGATION

2. MATERAIALS AND METHODS

Collection of plants

The leaf twigs of the plants *Annona squamosa, Annona reticulata and Annona muricata* were collected with the help of suitable instruments and temporarily stored in plastic bags to prevent desiccation and the protection of flowering parts. The specimens were deposited in the herbarium of PG Department of Botany and Biotechnology, Bishop Moore College, Mavelikara, Kerala. (BMC.B. 25, 26, 27).

Identification of plants

Identification of the plants under investigation was made with the help of suitable literature like Gambles's[16] flora of the presidency of Madras based on morphological and reproductive characters. Then leaves are carefully separated from the twigs and washed thoroughly with distilled water.

Extract preparation of plant materials

Identified materials are subjected to air dry and the dried specimen is grinded to form fine powder for the extraction of phytochemicals. For preparing aqueous leaf extract 4g of powdered dried and grinded leaves were taken into a beaker along with 40 ml of deionized water and was allowed to boil at 80^0C for 30 min in a water bath and subsequently cooled down to room temperature. For preparing 1:1 deionized water: methanol extract, 4g of samples was suspended in a solution containing 20ml of deionized water and 20ml of methanol. The prepared solutions were initially filtered with cheese cloth and subsequently twice using crude filter thereby powdered leaves materials were filtered out. The filtrates were again filtered twice through Whatman No 1 filter paper to get clear solution. The filtrate was stored at 4^0C till use.

Phytochemical analysis

The aqueous extracts of plants were subjected to phytochemical analysis using methodology of Sofowora[17] and Kepm[18]. The major pharmaceutically valuable phytochemical compounds investigated in the present study were Alkaloids, Carboxylic acids, Coumarins, Flavonoids, Phenols, Proteins and free amino acids, Quinones, Resins, Saponins, Sterols, phytosterols and triterpenoidal, sapogenins, Tannins, Xanthoproteins, Sugars and Glycosides

Synthesis of Copper Nanoparticles

10 ml solution of each leaf extracts were introduced drop wise into 10 ml of 10mM (0.01mM) solution of copper sulphate under continuous stirring[19] for 30 minute. After the complete addition of leaf extract, the mixture was kept for incubation for 24h in a dark atmosphere. A control set up was also maintained without adding copper sulphate to the plant extract.

UV-Visible Spectrophotometer analysis of nanoparticles

The formation of copper nanoparticle was also confirmed by spectrophotometric analysis. 100 µl of the sample was pipetted into a test tube and diluted with 10 ml of deionized water and subsequently analyzed at room temperature[20]. The analysis was done using UV-Visible spectroscopy, Jasco V- 550 spectrophotometer instrument. The reduction of copper nanoparticles was monitored by UV-Visible spectrophotometer at a range of absorbance from 200-500nm.

SEM analysis of nanoparticles

The morphology and size of the copper nanoparticles were investigated using Scanning Electron Microscope (SEM).

Antimicrobial activity of the leaf extracts

The bacterial strains are procured from Microbial Type Culture Collection (MTCC, Chandigarh, India) were employed in the present study to investigate the antibacterial properties. The gram negative organisms such as *Escherichia coli* (MTCC 585), *Pseudomonas aeroginosa* (MTCC2642) and Gram positive organisms such as *Bacillus subtilis* (MTCC 428), *Staphylococcus aureus* (MTCC 3160) were used as the test pathogens. The antimicrobial activity was assessed using the Agar well diffusion assay[21.] In brief, all bacterial cultures were plated out on Nutrient agar plates and were incubated for 24 h at 37 $\pm 0.5^0$C and colonies from this fresh culture were used for making suspension. Fresh inoculums of approximately 106 CFU (colony forming units/ml) of tested microorganisms were used for the study. 100 micro liter of the bacterial suspension was uniformly spread on sterile Muller Hinton Agar plates. After the solidification of agar, wells were made with a 6 mm sterile cork borer. 100 micro liter plant extracts, copper nano suspension and 10 micro liter Copper sulphate were poured in to the wells. The plates were incubated for 24 h at 37±0.5 °C and antibacterial activities of the extracts were observed by measuring the zone of inhibition in millimeters. Here antibiotic Gentamycin (25 mg) used as positive control at the center of the plate.

Effect of copper nanoparticles on root elongation

Seeds were immersed in a 5% sodium hypochlorite solution for 10 min to ensure surface sterility, and then they were soaked in distilled water or nanoparticle suspension or 10mM copper sulphate solution for about 6 hrs. After soaking the seeds were rinsed thoroughly with distilled water. To study the effect of copper nanoparticles on seed germination three layers of sterilized filter papers were fitted in 100 mm x 15 mm petri plates and soaked with different concentrations of distilled water or nanoparticle suspensions in water and Water:methanol or copper sulphate solution. Demineralized water and 10mM copper sulphate solution were used as control. Seeds were transferred on to the filter paper, with 15 seeds per dish and 1 cm or larger distance between each seed[22]. The covers of the petri dishes were closed and they were incubated at 25^0C for 5 days. The germination rate was recorded at the end of 5^{th} day. The parameters adopted in this analysis to evaluate the conditions of seed germination were Relative root germination rate and Germination Index. They were calculated based on the following equations:

$$\text{Relative root elongation} = \frac{\text{Mean root length in test sample X 100}}{\text{Mean root length in control}}$$

$$\text{Relative germination rate} = \frac{\text{Seeds germinated in test sample X 100}}{\text{Seeds germinated in control}}$$

3. RESULT AND DISCUSSION

The three samples *Annona squamosa L.*, *Annona reticulataL.* And *Annona muricata L.* belongs to the family annonaceae, order ranales and the series thalamiflorae seen are in different regions of tropical and subtropical areas (Fig – 1). Prior to using the aqueous extracts and aqueous extracts with methanol (1:1) of these plants for bio-nanosynthesis, the phytochemical evaluation of these plants was done.

PHYTOCHEMICAL ANALYSIS

The results of phytochemical analysis are given in tables 1 – 3. Table – 1 shows the phytochemical analysis of *Annona squamosa* L., and the following phytochemicals were present in both extracts (aqueous as well as methanol water mixture), Alkaloids, Flavonoids, Phenols, Quinones, Glycosides, Carotenoids, Sugar and Terpenoids, whereas, Coumarins, Saponins, Sterols, Tannins and Phlobatannins were only seen in aqueous extracts. Table – 2 represents the phytochemical analysis of *Annona muricata* L. extracts, Alkaloids, Flavonoids, Phenols, Quinones, Resin, Tannins and Sugar were present both the extracts. Table – 3 shows the phytochemical analysis of the extracts of *A. reticulata*. The phytochemicals present in both the samples were Alkaloids, Flavonoids, Sterols, Tannins, Xanthoproteins, Glycosides, Tannins, Diterpines and Carotenoids. The phytochemical study shows that leaf extracts contain Alkaloids, Flavonoids, Quinones, Phenols, Terpenoids etc. But do not contain Carboxylic Acids, Lignin, Proteins and Amino acids etc in various extracts. Presence of the phytochemical compounds reveals that, these three annonacean members have the tremendous biological activity.

The earlier studies on the phytochemical screening of leaf extract of *Annona squamosa* revealed that the presence of active ingredients such as Glycosides, Steroids, Saponins, Phenols, Flavonoids, Terpenoids and Tannins, while the leaf extract of *Annona reticulata* revealed that the presence of Alkaloids, Tannins, Steroids, Terpenoids and Coumarins[23]. Phytochemical analysis on the *Annona muricata* [24] revealed the presence of Tannins, Steroids and Cardiac Glycosides. These studies are in agreement with our findings. These phytochemicals in the plant extracts can reduce the metal ions to metal nanoparticles [25].

SYNTHESIS AND CHARACTERIZATION OF COPPER NANOPARTICLES

The aqueous leaf extracts of *Annona squamosa* L., *Annona muricata* L., *Annona reticulata* L. were used for this study. 10 ml of aqueous extract or aqueous leaf extracts with methanol in the ratio 1:1, were added drop by drop in to the 30 ml of 0.01M (10Mm) solution of copper sulphate under continuous stirring far 10 min. After the complete addiction of leaf extracts, the mixture was kept for incubation in dark for 24hours. The change in colour of reactants and an appearance of a colloidal nature in the solution indicates the presence of copper nano particles in solution.

CHARACTERIZATION OF COPPER NANO SUSPENSION

The following methods were used for the physical as well as biological characterization of copper Nanoparticles

UV-VIS SPECTRUM ANALYSIS

The reduction of pure Copper ions was monitored with UV-Vis spectrum of the reaction medium after diluting the aliquot of the sample with deionized water. The result obtained from UV-Visible spectroscopy analysis is presented in (Fig – 2). There is a peak can be seen at a range of 521 nm for all three samples. It is the clear evidence for the presence of nanoparticles.

SCANNING ELECTRON MICROSCOPY ANALYSIS

Particle size, distribution and morphology are the most important parameters of characterization of nanoparticles. Morphology and size are measured by electron microscopy. The Fig –3 shows the copper nanoparticles synthesized by the leaf extracts of *Annona squamosa, Annona muricata*, and*Annona reticulata*. The nanoparticles have a tendency to adhere strongly to each other, forming agglomerates. The SEM analysis was done using Hitachi model S-3400n SEM machine. Metal oxide nanoparticles often agglomerate in the aqueous phase to minimize surface energy [26]. In this study also the tested nanoparticles showed agglomeration.

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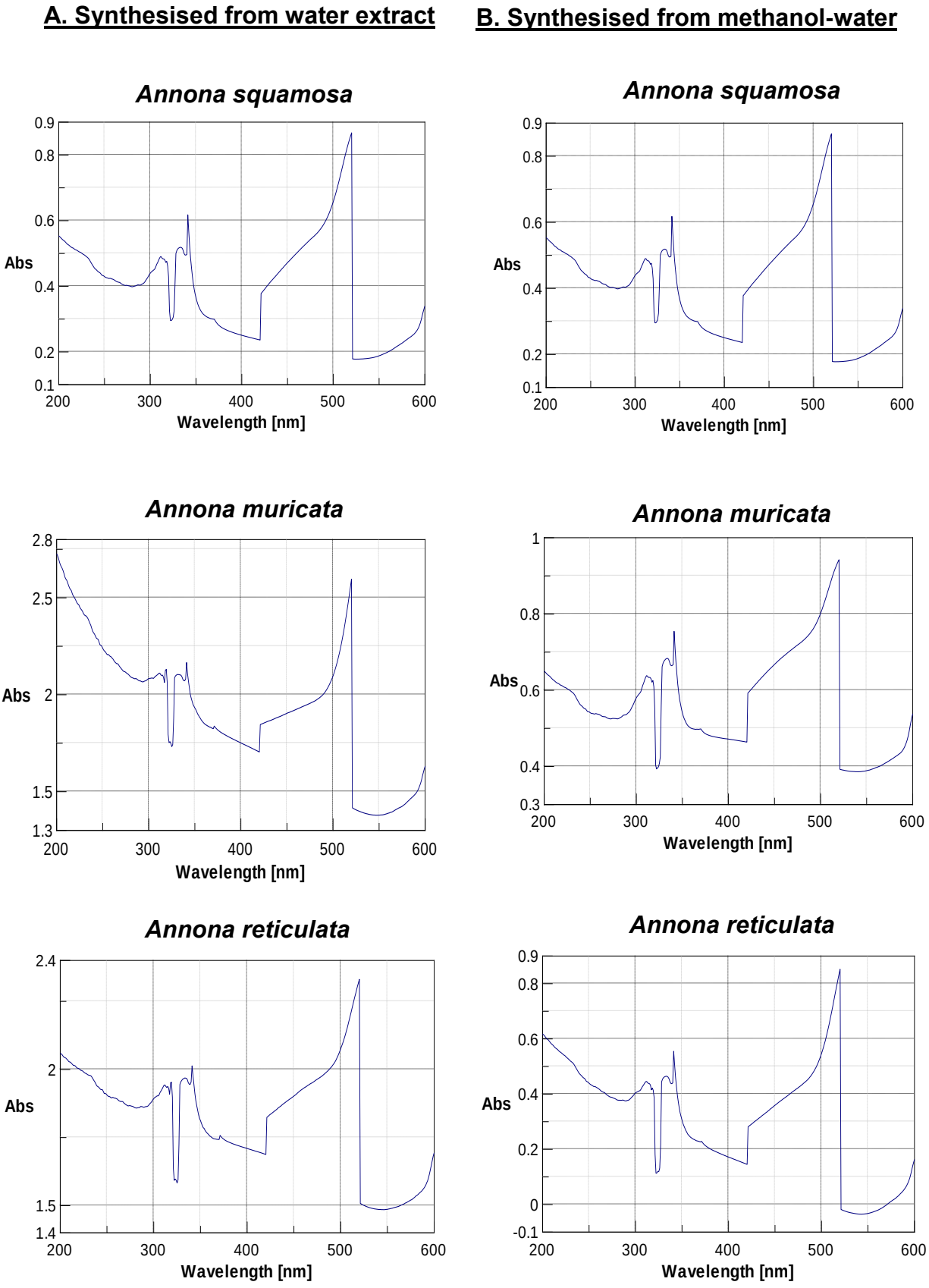

FIGURE 2: UV-VIS SPECTROPHOTOMETER ANALYSIS OF BIOSYNTHESIZED NANOPARTICLES

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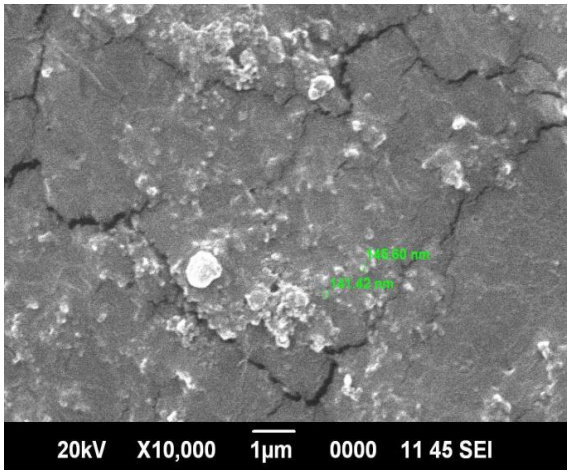
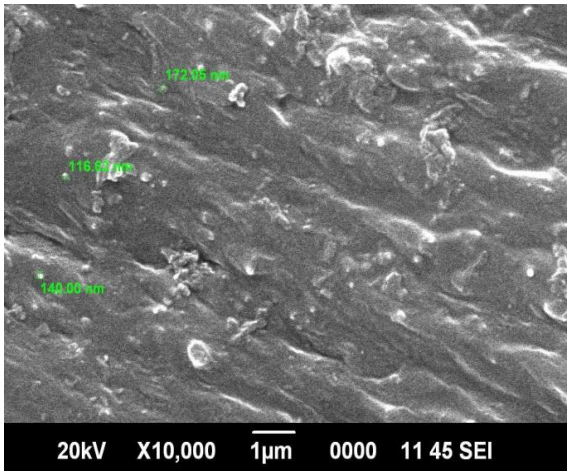
A. Synthesised from water extract

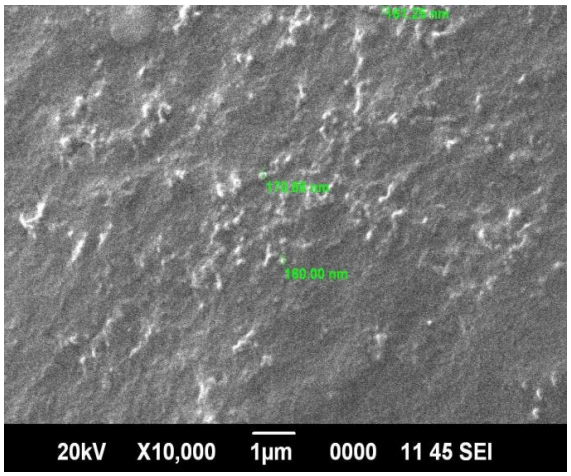
Annona squamosa



Annona muricata

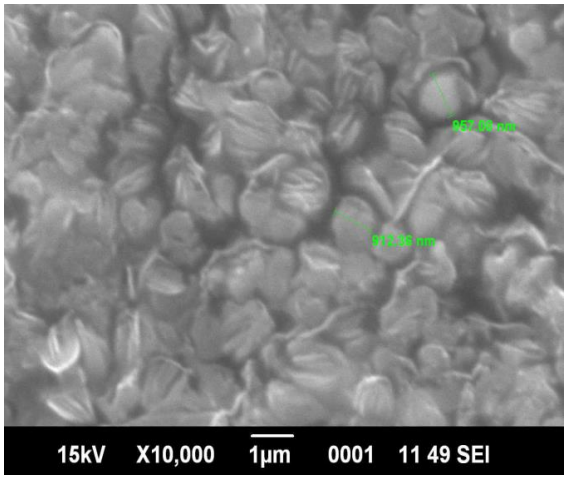


Annona reticulata

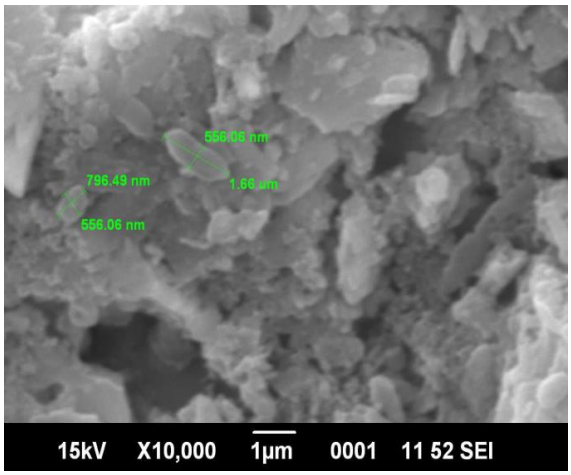


B. Synthesised from methanol-water

Annona squamosa



Annona muricata



Annona reticulata

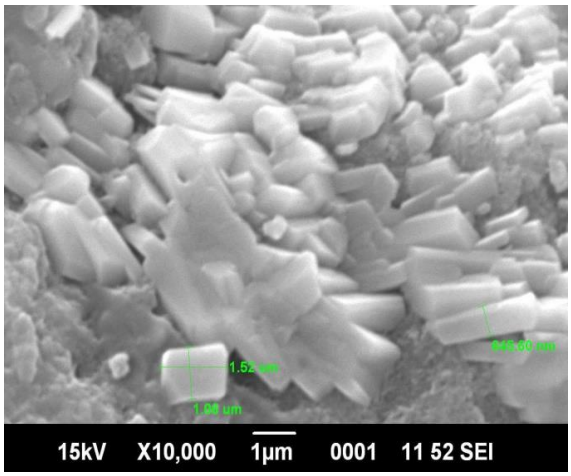


FIGURE 3: SEM MICROGRAPH OF COPPER NANOPARTICLES

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International Journal of Life Sciences Research ISSN 2348-3148 (online)
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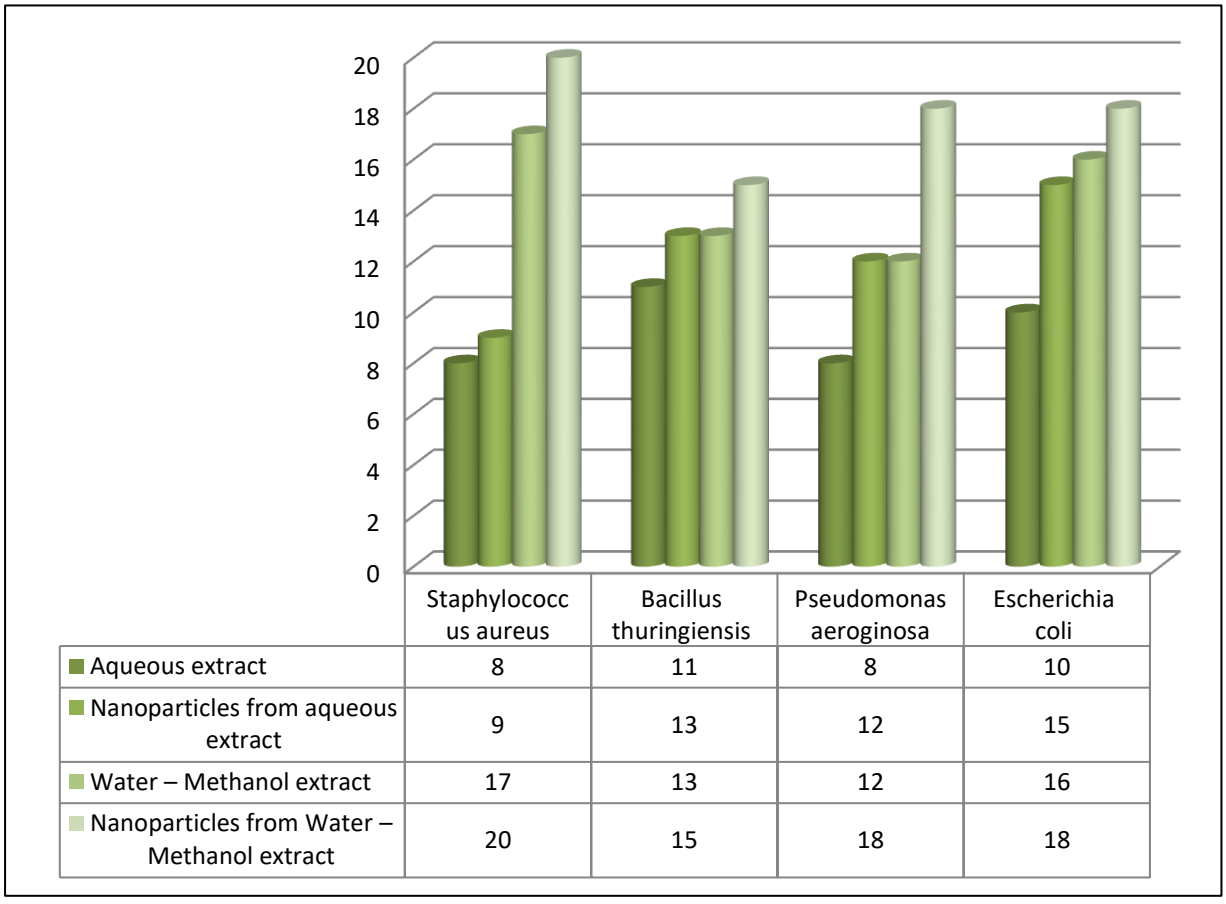
ANTIMICROBIAL ACTIVITY

The antimicrobial effect of biologically synthesized copper nanoparticles was analyzed against four bacterial strains, *Escherichia coli*, *Pseudomonas aeroginosa* (both Gram negative) and *Bacillus subtilis*, *Staphylococcus aureus* (both Gram positive). In this experiment antibiotic Gentamycin was used as positive control. 10mM copper sulphate solution was used as the negative control and no zone of inhibition was observed against copper sulphate. (The zones of inhibitions were evaluated in mm.) The antimicrobial activities of the extracts and the nanoparticles prepared form them were evaluated and the results are summarized in Fig. –4 to 6.

Fig.–4 denotes the antimicrobial activity of nanoparticles and leaf extracts of *Annona squamosa* against various bacterial strains. The results of the experiment indicate that the nanoparticles synthesized from Water:Methanol (1:1) mixture extract shows the highest activity against *Staphylococcus aureus* and least activity shown in aqueous extract. The antimicrobial activity of *Annona reticulata* is shown in Fig. –5. In this part of the study it was observed that Nanoparticles synthesized from Water:Methanol mixture extract of *Annona reticulata* exhibited the highest antimicrobial activity than nanoparticles synthesized form aqueous extract alone. *Staphylococcus aureus* displayed the highest antimicrobial activity in nanoparticles of water: mehanol extract. Fig. – 6 represent the antimicrobial activity of *Annona muricata*. In this part of the experiment the highest activity was observed against *Pseudomonas aeroginosa*in nanoparticles of water:methanol extract. On the other hand least activity shows in aqueous extracts against two bacteria *Bacillus thuringiensis* and *Pseudomonas aeroginosa*.

From the study it is clear that all the three samples *Annona squamosa*, *Annona muricata* and *Annona reticulata* show good antimicrobial activity against both gram positive and gram negative bacteria. The nanoparticles synthesized from water-methanol mixture exhibited higher antimicrobial activity than nanoparticles synthesized from water extract alone. The highest antimicrobial activity was shown by *Annona squamosal* and *Annona reticulata* against *Staphylococcus aureus*, while *Annona muriata* exhibited the highest activity against *Pseudomonas aeroginosa*.



	Staphylococcus aureus	Bacillus thuringiensis	Pseudomonas aeroginosa	Escherichia coli
Aqueous extract	8	11	8	10
Nanoparticles from aqueous extract	9	13	12	15
Water – Methanol extract	17	13	12	16
Nanoparticles from Water – Methanol extract	20	15	18	18

Figure 4: Antimicrobial activity of Annona squamosa L.

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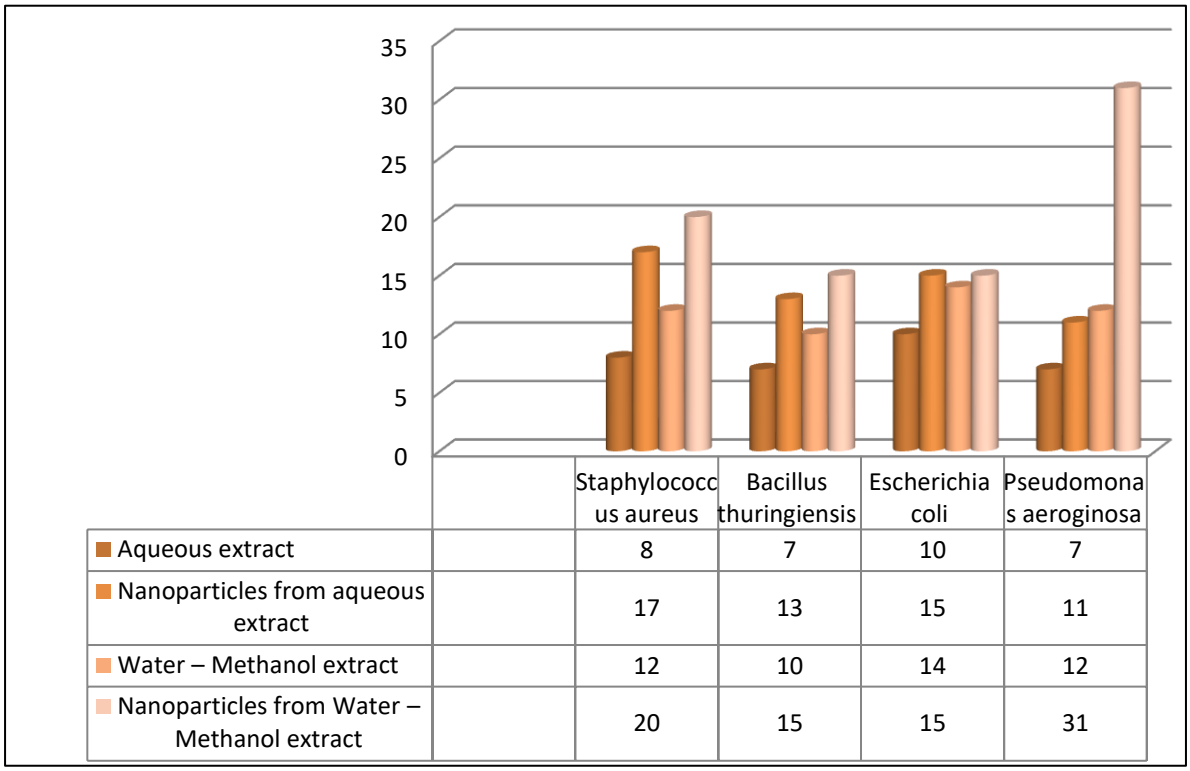
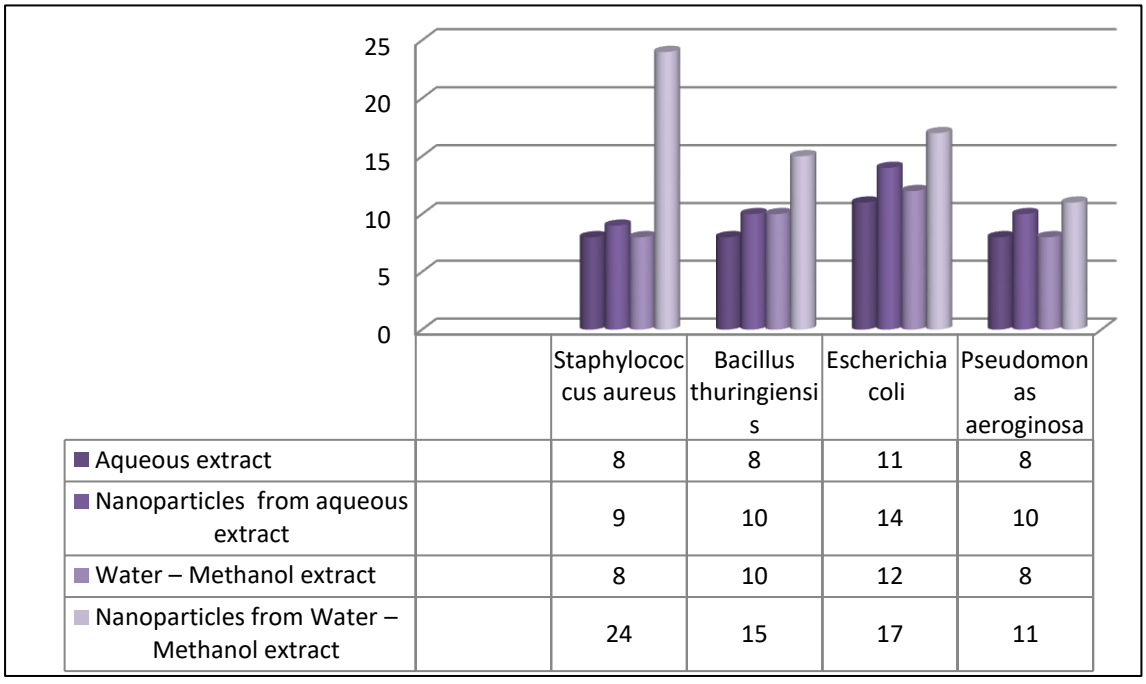


	Staphylococcus aureus	Bacillus thuringiensis	Escherichia coli	Pseudomonas aeroginosa
Aqueous extract	8	7	10	7
Nanoparticles from aqueous extract	17	13	15	11
Water – Methanol extract	12	10	14	12
Nanoparticles from Water – Methanol extract	20	15	15	31

Figure 5: Antimicrobial activity of Annona muricata L.



	Staphylococcus aureus	Bacillus thuringiensis	Escherichia coli	Pseudomonas aeroginosa
Aqueous extract	8	8	11	8
Nanoparticles from aqueous extract	9	10	14	10
Water – Methanol extract	8	10	12	8
Nanoparticles from Water – Methanol extract	24	15	17	11

Figure 6: Antimicrobial activity of Annona reticulata L

SEED GERMINATION ASSAY

Metal nanoparticles are considered to modify physiological and biochemical processes in plants thereby affecting their germination and growth favorably or otherwise[27] .This assay was carried out to study the effect of copper nanoparticles in seed germination and root elongation of green gram. Relative root length was calculated by comparing the seeds treated using various plant extracts and the copper nanoparticles with the corresponding control seeds, treated only with deionized water and with 0.01M copper sulphate solution. The results of this study are summarized in fig. – 7.

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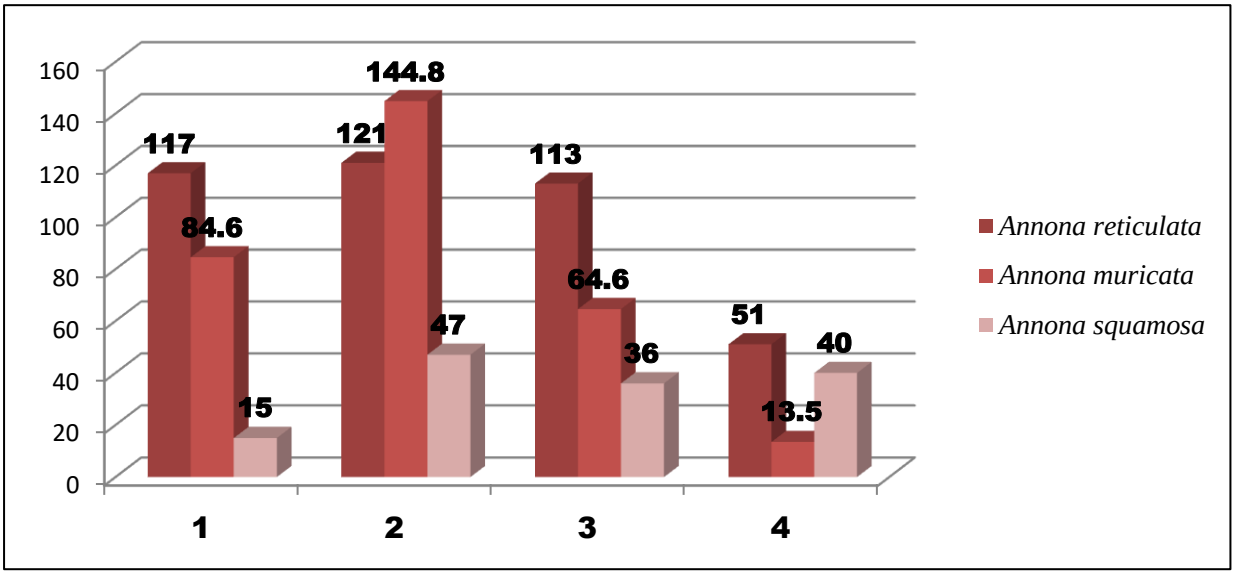

1. **Aqueous extract**
2. **Nanoparticles from aqueous extract**
3. **Water – Methanol extract**
4. **Nanoparticles from Water – Methanol extract**

Figure 7: ROOT ELONGATION RELATIVE TO CONTROL

Relative root elongation in green gram was studied with *Annona reticulata*, *Annona muricata* and *Annona squamosa* nanoparticles leaf extracts. The nanoparticles from aqueous extracts exhibited greater root elongation than their corresponding aqueous extracts alone. Also study reveals that water: methanol leaf extract and nanoparticles synthesised from water: methanol extracts show least activity in root elongation. This points out to the fact that the nanoparticles did not affect the germination of green grams adversity, even though there is a difference in the germination between the nanoparticles and their corresponding extracts.

The germination index was studied by calculating the relative germination rate. All the extract treatments led to the germination of green gram seeds. In the current study all the treatments led to 100% germination of seeds, the results corroborated by Lin and xing (2007)[28] who reported that nano-ZnO was not affected seed germination of radish, rape, ryegrass, lettuce and cucumber except the corn seed.

Relatively fewer studies have been reported on application of copper nanoparticles. Adhikari et al.,[29] showed that all the treatments with nano-CuO particles in the seeds of soybean and chickpea led to 100% germination of seeds and according to them this indicated that there is no adverse effect of nano-CuO particles in seed germination. This result is in agreement with our findings. Shah and Belozerova[30] observed favorable effect of Copper nano particles on germination of lettuce seeds is in agreement with our results. Yasmeen *et al.* [31] reported that, the effect of copper nanoparticles on seed germination depends on the treatment methods.

According Naik and Lakshmi (2018)[32] seed germination studies can be used as an alternative to animal model to study bioassay principles. In this study, we observed hundred percent seed germination in green gram, in all the nine extracts as well as in the nanoparticle suspensions. However, the root growth index varied among the nanoparticle suspensions. This shows that copper nanoparticles did not adversely affected the germination of seeds under investigation.

4. CONCLUSION

Present study reveals the presence of various phytochemicals in the various leaf extracts of *Annona squamosa*, *Annona reticulate* and *Annona muricata.* This investigation further illustrated that the extracts of the above mentioned plants have the capacity to reduce copper into copper nanoparticles. We also investigated the antimicrobial activity of nanoparticles against both gram positive and negative bacteria and study proved that nanopartices have the good bactericidal capacity;

however nanoparticles from water-methanol mixture exhibited higher antimicrobial activity. Results from the seed germination assay indicate that biologically synthesized copper nanoparticles are nontoxic to higher plants. These results taken together points to the fact that the biologically synthesized copper nanoparticles are potent antimicrobial agents and they can be used for the formulation of new bactericidal materials. This study is of importance owing to the fact that the method used here is economically viable and is environmental friendly.

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