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Green synthesis of copper and zinc nanoparticles from *Glycosmis pentaphylla* and *Azadirachta indica* and evaluation of their antifungal activity against *Fusarium oxysporum cubense*

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Abstract
Soil borne diseases which are caused to various plants include a wide variety of soil microbes like fungi and bacteria, among which Fusarium wilt is one such disease caused by *Fusarium oxysporum cubense* in banana plants. Wilt disease or the panama disease of plant is among the most destructive disease of banana in the tropics and even the control methods like field sanitation, soil treatments and crop rotations have not been a long term control for this disease. An alternative method of treating *Fusarium oxysporum* was adopted by using various natural plant leaves of *Glycosmis pentaphylla* and *Azadirachta indica*. Nanoparticles are small particles with a dimension of 10^{-9} and 10^{-10}. Green synthesis is a new method developed for the synthesis of nanoparticles which is small in size, large surface area and eco- friendly. Leaf extracts of these plants were used for synthesis of copper and zinc nanoparticles, as nanoparticles are powerful antimicrobial agents. The extract is prepared with a stock solution of 100mM copper sulphate and 100mM zinc sulphate. The leaf extracts were prepared with 5 solvents (Distilled water, Propane, Hexane, Acetone and Methanol). The action of plant leaves were observed by the zone of inhibition obtained with a concentration of 50, 100 and 150µl respectively. The result was more in copper nanoparticles of leaf extract as compared to the zinc nanoparticles of particular leaf extracts but the zinc particles with methanol and propane showed good result with particular leaves. In dried condition of leaves copper nanoparticles with propane as solvent exhibited a greater zone of inhibition. Moreover the solvent, methanol showed good results with both zinc and copper nanoparticles. The synthesized nanoparticle were characterized by UV-VIS spectrophotometry to confirm the formation of nanoparticles. Green synthesis is used namely because of low cost, simple, use of less toxic materials, most important is eco-friendly.

Keywords: Nanoparticles, *fusarium oxysporum cubense*, panama wilt, *glycosmis pentaphylla*, *azadirachta indica*, antifungal assay, PDA

1. Introduction
Nanotechnology is an emerging field of science. It has increased applications in diverse area for the development of new materials at nanoscale levels (Paul *et al*., 2015) [61-62]. Nano-technology mainly consists of the processing of, separation, consolidation, and deformation of materials by one atom or one molecule (Prasad *et al*., 2008) [67-68]. Nanoparticles has 1-100 nm in size and they possess novel physical and chemical properties (Sajeshkumar *et al*., 2015a; Sajeshkumar *et al*., 2015b) [74-75]. Nanoparticles bear antibacterial properties (Hajipour *et al*., 2012) [26].

Nanoparticles play important role in fighting against disease causing microbes. Nanoparticles are very minute particles. Due to large surface volume ratio; renewable surface and varying micro electrode potential values nanoparticles are largely used as catalysts also (Din and Rehan, 2017) [11]. There are different types of nanoparticles including; silver, copper, zinc (metal nanoparticles).

Nowadays humans face dangers infections due to pathogenic microbes. Nanoparticles can overcome this problems. Nanoparticles have antibacterial property. Metal nanoparticles such as silver, copper and zinc has inhibitory effect on microorganisms.

Nanotechnology is an emerging field which makes an impact on human life such as health, food, chemical and energy industries, environmental and space industries etc.

Various methods to synthesize nanoparticles include sol gel method, chemical reaction, solid state reaction and co precipitation. Another method used is the green synthesis method which is one of the most appropriate method used in recent years. This method have several advantages namely low cost, simple, use of less toxic materials, most important is eco-friendly. The metal nanoparticles such as Ag, Cu etc., are found to have antibacterial and antifungal activity. This effect of metal nanoparticles has been attributed to their small size, and high surface to volume ratio, which allow them to interact closely with microbial membranes and it is not merely due to the release of metal ions in solutions. The antibacterial and antifungal properties of the metal nanoparticles find applications in various fields such as medical instrument, and devices, water treatment and food processing. Some of the methods to prepare nanoparticles is by using the methods such as thermal reduction, vacuum vapor deposition, microwave irradiation methods, chemical reduction, and laser ablation. All these methods use oxygen-free atmosphere to synthesize copper, zinc or aluminium nanoparticles because it easily oxidizes. Nanoparticles have various applications in optoelectronics, nanodevices, nanoelectronics, nanosensors, information storage etc. Among various metal particles, copper nanoparticles have attracted more attention because of their catalytic, optical, electrical and antifungal/antibacterial applications (Ramyadevi *et al*., 2012) [73].

Soil borne diseases which are caused to various plants include a wide variety of soil microbes like fungi and bacteria, among which Fusarium wilt is one such disease caused by *Fusarium oxysporum cubense* in banana plants. A decrease in the pathogen growth in soil is manipulated through agro-ecosystem, which focuses on the depletion of various soil borne diseases in banana plants (Shen *et al*., 2019) [78]. Bananas are an important source of living for farmers across wet tropics and subtropics, including various countries like Americas, Africa, Southeast Asia and the Pacific. Although it is a commercial crop in the world, but it is considered that 87% of the banana production is for local food consumption (Langhe, *et al*., 2009) [9].

Wilt disease or the panama disease of plant is among the most destructive disease of banana in the tropics and even the control methods like field sanitation, soil treatments, crop rotations and organic amendments have not been a long term control for this disease. Many potential biocontrol agents can be developed against *Fusarium oxysporum cubense* by understanding the interactions between the biocontrol and fungal pathogen (Getha and Vikineswary, 2002) [20]. Bananas are rich sources of both simple and complex carbohydrates, and of the vitamins ascorbic acid, B6, carotene, niacin, riboflavin, and thiamin. They are also excellent sources of potassium. Moreover, bananas are easily digestible, offering access to food energy faster than apples and meats.

The reddish brown discoloration of the xylem, develops in feeder roots, the initial sites of infection shows the first internal symptom. Vascular discoloration progresses to the rhizome, is most prominent where the stele joins the cortex. The younger leaves wilt and collapse until the entire canopy consists of dead or dying leaves. *F. oxysporum* cannot be morphologically distinguished easily. There colonies grow 4-7 mm on potato dextrose agar at 25 °C with abundant white to purple mycelium. (Ploetz, 2005) [65] Both pathogenic and nonpathogenic strains of *F. oxysporum* are found in agricultural soils throughout the world, and it is these populations that have received the most attention from researchers. It is not a pathogen of plants in native situations and the grasslands often support large populations of *F. oxysporum*, yet grasses, whether cultivated or native, are not known to be hosts to pathogenic strains of this fungus. Moreover understanding the evolution of pathogens in *F. oxysporum* species will ultimately require a detailed characterization of the relationships between diverse pathogenic and nonpathogenic forms in this species (Gordon and Martyn, 1997) [22].

1.1 Green synthesis of nanoparticles

Synthesis of nanoparticles using biological methods is referred as greener synthesis of nanoparticles. Green synthesis provides advancement over chemical and physical method as it is cost effective, environment friendly, and safe for human therapeutic use (Kumar *et al*., 2009). Metals like silver, copper and zinc has inhibitory effect on microbes. Biological synthesis of metallic nanoparticles is inexpensive single step and eco-friendly methods. The plants and seeds are used successfully in the synthesis of various greener nanoparticles such as copper, silver, and zinc oxide (Kuppusamy *et al*., 2014; Mishra *et al*., 2014).

1.2 Application of nanoparticles

Nanoparticles has various applications. Nanoparticles have been used for constructing electrochemical and biosensors (Luo *et al*., 2006). Metal nanoparticles embedded paints have good antibacterial activity (Kumar *et al*., 2008) [41]. Current research is going on regarding the use of magnetic nanoparticles in the detoxification of military personnel in case of biochemical warfare (Salata, 2004).

One of the major opportunities for nanoparticles in the area of computers and electronics is their use in a special polishing process, chemical-mechanical polishing or chemical mechanical planarization, which is critical to semiconductor chip fabrication (Elechiguerra *et al*., 2005) [15].

Magnetic nanoparticles are also used in targeted therapy where a cytotoxic drug is attached to a biocompatible nanoparticle for tumour cell treatment (Pankhurst *et al*., 2003) [59]. Porous nanoparticles have been used in cancer therapy. Bioremediation of radioactive wastes from nuclear power plants and nuclear weapon production such as uranium has been achieved using nanoparticles (Duran *et al*., 2007) [13].

1.3 Copper nanoparticles

Copper nanoparticles have high optical, catalytic, mechanic and electrical properties. They are cheap high yielding and have short reaction time under normal reaction condition. Copper nanoparticles have anti-microbial activities against various bacterial and fungal strain from any researchers (Patravale *et al*., 2004) [60]. It is used in various fields including agricultural, industrial, engineering and technical fields. Effective anti-bacterial activities are exhibited by copper nanoparticles. They are cost effective and have efficient bio synthesize techniques. Copper nanoparticles have less cost than silver and gold nanoparticles.

1.4 Zinc nanoparticles

Zinc nanoparticles have wide application; various synthetic methods have been employed to produce ZnNps (Chen *et al*., 2007) [6]. Zinc nanoparticles can produced from zinc oxide and zinc sulphate. Zinc nanoparticles has several medicinal uses, which harm skin, stomach, intestine and lymphastic system and they probably induces tumours. Zinc nanoparticles

has antibacterial effect on microbes, and it mainly depends up on the size and the presence of visible light. Zinc nanoparticles are used in the optical devices, sensors, catalysis, biotechnology, DNA labelling, drug delivery, medical, chemical and biological sensors (Devasenan *et al*., 2016) [10].

1.5 Antimicrobial activity
Anti-microbial agent is a substance that kills microorganisms or stops their growth. Anti-microbial medicines are grouped according to the micro-organisms they act. Antibiotic are used against bacteria, antifungal are used against fungi. They are also classified on the basis of their function. The agents that kill microbes are called microbicidal; those that merely inhibit their growth are called biostatic (Al Juhaiman *et al*., 2010) [1]. The use of anti-microbial agents for the treatment of infection is known as anti-microbial therapy. The use of antimicrobial medicines for the prevention of infection is known as antimicrobial prophylaxis.

1.6 Antifungal activity
Antifungals are used to treat fungal infections. The drug toxicity to humans and other animals from antifungals is generally high. They comprise a large and diverse group of drugs used to treat fungal infections. The mechanism of action of the antifungals include inhibition of fungal membrane and cell wall synthesis, alterations of fungal membranes, effects of microtubules and inhibition of nucleic acid synthesis. Antifungal activities potentially offer solution to the problem of antibiotic resistance. The antifungal medication is also called as antimycotic medication, a pharmaceutical fungicide used to treat and prevent mycosis and serious systematic infections. They are made to acts against plants, animals and humans. The modern era of antifungal therapy by the introduction of oral griseofulvin and tropical chlormidazole in1958 and the subsequent introduction of IV AmB in 1960. Antifungal creams, liquids and sprays are used to treat fungal infections.

1.7 Agar well diffusion
Agar well diffusion test is used for antifungal assay. The well that cut on the solidified agar act as pour for loading sample. The agar that is inoculated with test organism after overnight incubation may show zone of inhibition. The sample that is diffused in the agar inhibits the growth of microbes.

1.8 Objectives
Synthesis of copper and zinc nanoparticles using leaf extracts of Pannal (*Glycosmis pentaphylla*), Neem (*Azadirachta indica*), and determine the antifungal properties of these nanoparticles against *Fusarium oxysporum cubense*.

1.9 Scope of the study
The study would enlighten the medical and pharmaceutical applications various green synthesised nanoparticles applications against *Fusarium oxysporum cubense* which could be further explored.

2. Review of literature
Copper nanoparticles widely used due to their superior, optical, electrical, antifungal/antibacterial and biomedical applications. Copper nanoparticles have superior antibacterial activity as compared to silver nanoparticles. Because copper is highly toxic to microorganisms (Singh, 2017) [81].
The antimicrobial activity mainly tested for drug discovery and prediction of therapeutic outcome. Agar disc diffusion and agar well diffusion are two methods used to evaluate antimicrobial activity (Balouiri *et al*., 2016) [2].

2.1 *Glycosmis pentaphylla* (Pannal)
Glycosmis pentaphylla is a species of flowering plant in the citrus family, Rutaceae, known commonly as orange berry and gin berry. It is an aromatic shrub or small (1.5–5.0 m) tree widely distributed from India, Malaysia and Southern China to the Philippine Islands where it occurs in tropical forests at low altitudes and has nearly 11 species. It is traditionally used for the treatment of fever, liver complaints and other diseases. The stems are widely used as a brush for cleaning the teeth and has various antimicrobial, antioxidant and cytotoxic activities. (Howlader, Rizwan *et al*., 2011) [28]
The whole plant is beneficial in anemia, jaundice, rheumatism, and cough. The leaf juice is used as an anthelmintic, febrifuge, vermifuge, in liver complaints, and applied in the form of paste to eczema and other skin diseases. Wood brushed with water is administered internally as an antidote for snakebite. The plant is also orally taken during the time of jaundice in traditional Indian medicines. The hepatoprotective activity of the stem and leaf water extract of this plant against carbon tetrachloride induced liver toxicity. (Nayak, Jain *et al*., 2010) [56].

2.1.1 Taxonomical classification (Pannal, Orange berry, Gin berry)
Kingdom: Plantae-Planta, plantes, plants, vegetal
Subkingdom: Viridiplantae
Super division: Embryophyta
Division: Tracheophyta
Class: Magnoliopsida
Order: Sapindales
Family: Rutaceae
Genus: Glycosmis
Species: *Glycosmis pentalphylla*.

2.2 *Azadirachta indica* (Neem)
Azadirachta indica is well known as a medicinal plant in India and many other surrounding countries and consists of a wide spectrum of biological activities. It is an evergreen plant. Every part of the tree is used for treatment as traditional medicines against human ailments. It has been extensively used in homeopathy, unani and Ayurveda and has become a part of modern medicine as well. (Biswas Kausik, Chattopadhyay *et al.*, 2002) [3]. It grows in poor nutrient soils in arid habitats and its long deep root system has the ability to extract nutrients and moisture from leached sandy soils and are capable to withstand drought but cannot handle water logged soils.
In India, flowering of neem plants occur around January to April. Neem woods are highly resistant to various pests and fungal attacks. Neem oils are highly rich in fatty acids and its extracts can inhibit the growth of fungus on animals. It possess pesticide property which involves a practice of adding dried neem leaves to stored grains or place with warm clothes to repel insects. Used in the manufacture of soaps, tooth powder, tooth paste, shampoos etc. (Koul, Isman and Ketkar, 1990) [37].]

2.2.1 Taxonomical classification (*Azadirachta indica*; Neem)
Kingdom: Plantae-- planta, plantes, plants, vegetal
Subkingdom: Viridiplantae

Superdivision: Embryophyta
Division: Tracheophyta
Class: Magnoliopsida
Order: Rutales
Family: Meliaceae
Genus: Azadirachta
Species: *Azadirachta indica*

Table 1: Different vernacular names of Pannal (*Glycosmis pentaphylla*) around the globe and India.

Language	Names
Scientific names	*Glycosmis pentaphylla*
Name in various global languages	
French	
German	Mangopfaume
English	Orangeberry/Gin berry
Name in various Indian languages	
Sanskrit	Ashbashakota
Hindi	Ban nimbu
Urdu	
Marathi	Kirmira
Kannada	Guruvade
Gujarati	
Malayalam	Pannal
Tamil	Kattu konchi

Table 2: Different vernacular names of Neem (*Azadirachta indica*) around the globe and India.

Language	Names
Scientific names	*Azadirachta indica*
Name in various global languages	
French	
German	
English	Neem
Name in various Indian languages	
Sanskrit	Pakvakrita
Hindi	Neem
Urdu	Neem
Marathi	Nimbay
Kannada	Turakabevu
Gujarati	Dhanujhada
Malayalam	Aryaveppu
Tamil	Veppai

3. Hypothesis

The current research work is based on the following hypothesis

1. Plant extracts of Pannal (*Glycosmis pentaphylla*) and Neem (*Azadirachta indica*), could be used as antifungal agents.
2. These plant extracts could be used in formulating nanoparticles (copper and zinc) and their antifungal activity vary widely.

4. Materials and Methods

4.1 Study area

Kerala state covers an area of 38,863 km^2 with a population density of 859 per km^2 and spread across 14 districts. The climate is characterized by tropical wet and dry with average annual rainfall amounts to 2,817 ± 406 mm and mean annual temperature is 26.8 °C (averages from 1871-2005; Krishnakumar *et al.*, 2009) [39]. Maximum rainfall occurs from June to September mainly due to South West Monsoon and temperatures are highest in May and November.

4.2 Sample collection

Samples of Pannal (*Glycosmis pentaphylla*), Neem (*Azadirachta indica*), were collected from Ramapuram, Kottayam district of Kerala State, India. The leaves were thoroughly cleaned using double distilled water. The samples were dried in hot air oven at 60 ºC for 48 hrs. and later stored in air tight polyethylene zipper bag for analysis.

4.3 Extraction method

4.3.1 Dried extraction

About 1 g of dried samples are taken in a test tube to which 9ml of distilled water, propane, hexane, acetone or methanol is added. The mixture is mixed well and is kept for half an hour. It is then filtered using a filter paper into a container which is then stored at 4 °C for further use. The obtained dried leaf extract shows different colour in different solvents.

4.3.2 Fresh extraction

Leaf extract is prepared with 10 g of fresh leaves (*Glycosmis pentaphylla*, *Azadirachta indica*) thoroughly washed with tap water and then with DH_2O and cut into small pieces. It is then crushed in a pistil and motor by adding 30 ml of DH_2O. It is then filtered using a filter paper into a container and is then stored at 4 °C for further use.

4.4 Synthesis of nanoparticles

4.4.1 Copper nanoparticles

The stock solution is prepared by dissolving 2.49 g of Copper sulphate ($CUSO_4$) in 100 ml of DH_2O. Add 9ml of the 100mM $CUSO_4$ solution to 1ml of the leaf extract and is allowed to react in room temperature. The copper nanoparticles will be formed after 2-3 hours.

4.4.2 Zinc nanoparticles

The stock solution is prepared by dissolving 2.87g of Zinc sulphate ($ZnSO_4$) in 100ml of DH_2O. Add 9ml of the 100mM $ZnSO_4$ solution to 1ml of the leaf extract and is allowed to react in room temperature. The Zinc nanoparticles will be formed after 2-3 hours.

4.5 Test microorganisms

Fusrium oxysporum cubense is a fungal plant pathogen that causes Panama disease of banana (Musa spp.), also known as fusarium wilt of banana. The test organism were obtained from the department of Pathology, Indian Council of Agricultural Research (ICAR), New Delhi.

4.6 Solvents

4.6.1 Distilled water

The water that has been boiled into vapour and condensed back into liquid in a separate container. Impurities in the original water that do not boil below or at the boiling point of water remain in the original container. Thus, distilled water is one type of purified water. It goes through a distillation process.

4.6.2 Propane

It is a three-carbon alkane with the molecular formula C_3H_8. Its boiling point is -42 °C and its melting point is-188 °C and has a molecular mass of 44.1 g/mol.

4.6.3 Hexane

It is an alkane of six carbon atoms, with the chemical formula C6H14. They are color less liquids, odor less when pure, with boiling points between 50 and 70 °C (122 and 158 °F). It is a good solvent if trying to dissolve a non-polar compound. I t is highly flammable and its vapours can be explosive.

4.6.4 Acetone
It is an organic compound with the formula $(CH_3)_2CO$. It is a color less, volatile, flammable liquid and is the simplest and smallest ketone. It is a good solvent for many plastics and some synthetic fibers and used for thinning polyester resin, cleaning tools etc. It is used as one of the volatile components of some paints and varnishes.

4.6.5 Methanol
It is the simplest alcohol, consisting of a methyl group linked to a hydroxyl group. It is a light, volatile, color less, flammable liquid with a distinctive odour. It is however far more toxic than ethanol. At room temperature, it is a polar liquid. It can be used as an antifreeze, solvent, fuel, and as a denaturant for ethanol.

4.7 Characterization of nanoparticles
4.7.1 UV-Vis spectroscopy
The periodic scans of the optical absorbance between 345 and 700 nm with a UV- Vis spectrophotometer (Model 118, Systronics, Mumbai, India) at a resolution of 1 nm were performed to investigate the reduction rate of green synthesised nanoparticles. Deionised water was used to adjust the baseline.

The reduction of Cu^{2+} and Zn^{2+} was monitored periodically by using a UV- Vis Spectrophotometer and the UV- Vis spectra of the reaction solutions were measured in the range of 375–760 nm.

4.7.2 SEM-EDX analysis
SEM-EDX Analysis was carried out in instrument JSM 6390 with acceleration voltage 20 kV. SEM reveals information about the sample including external morphology, chemical composition and crystalline structure and orientation of materials making up the sample. SEM provides detailed high-resolution images of the sample by rastering a focused electron beam across the surface and detecting secondary or back scattered electron signal. The EDX spectrum of the silver nanoparticles was performed to confirm the presence of elemental silver signal and provides quantitative compositional information.

4.8 Antifungal assay
Antifungal assay was performed by agar well diffusion method. Active cultures of *Fusarium oxysporum cubense* were aseptically swabbed on Potato Dextrose Agar (PDA) plates using sterile cotton swabs. Wells of 7 mm diameter were made in the inoculated plates using sterile syringe (with front end cut and polished) and wells are filled with 50, 100 and 150 µl of nanoparticle solution, control (stock solution) and sample (fresh and dry leaf extracts). The plates were incubated at 25 °C for 72 hours after which the diameter of zones of inhibition were measured on regular intervals (24, 48 and 72 hrs.).

4.9 Statistical analysis
The results were analyzed and descriptive statistics were done using SPSS 12.0 (SPSS Inc., an IBM Company, Chicago, USA) and graphs were generated using Sigma Plot 7 (Systat Software Inc., Chicago, USA).

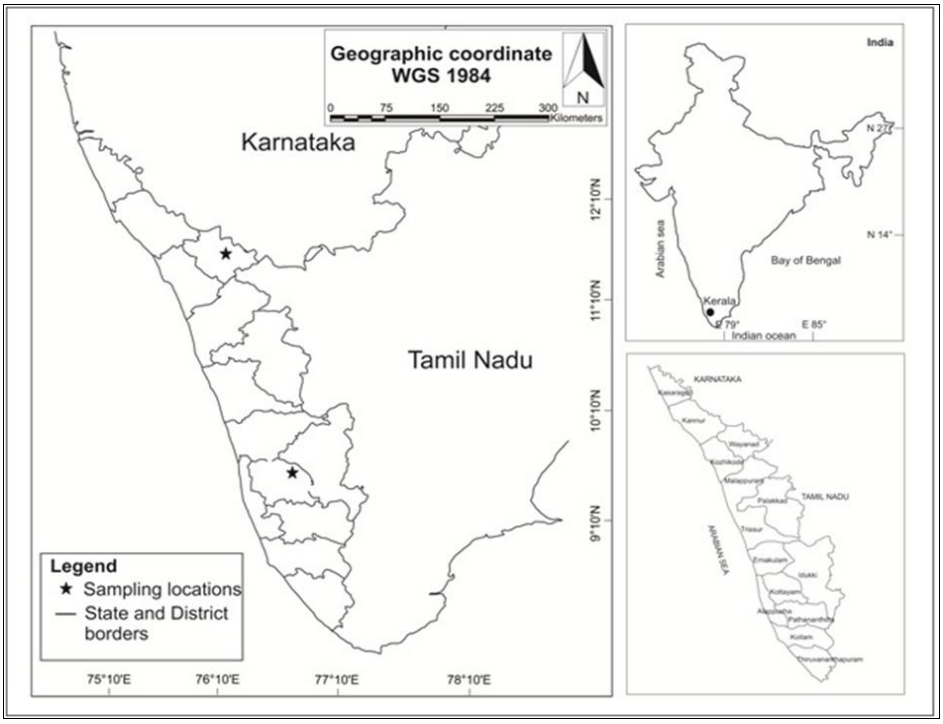

Fig 1: Map of Kerala showing the sample collection point.

Fig 2: Pannal (*Glycosmis pentaphylla*), Description of a) tree in natural habitat, b) fully developed berries, c) partially mature berries, d) developing flower inflorescence. Photo courtesy: Wikipedia

Fig 3: Neem (*Azadirachta indica*) description a) plant with leaves and flowers, b) plant with leaves, c) partially mature fruits, d) flowers, e) cleaned seeds ready for sale, f) cleaned seeds. Photo courtesy: Wikipedia.

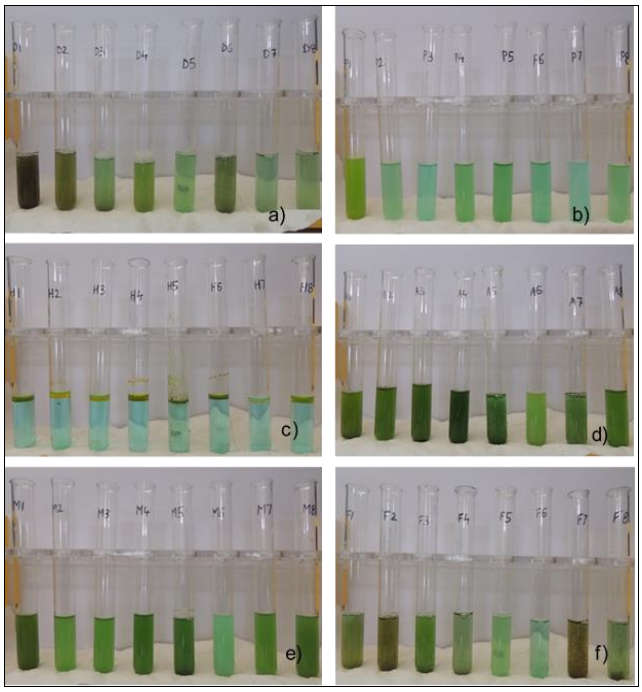

Fig 4: Green synthesised Copper nanoparticles of *Chromolaena odorata*, *Justicia adhatoda*, *Glycosmis pentaphylla*, *Azadirachta indica*, *Gliricidia sepium*, *Piper nigrum*, *Ocimum tenuiflorum* and *Tabernaemontana divaricate* a) dry leaf distilled water extracts, b) dry leaf propane extracts, c) dry leaf hexane extracts, d) dry leaf acetone extracts, e) dry leaf methanol extracts, f) fresh leaf distilled water extracts.

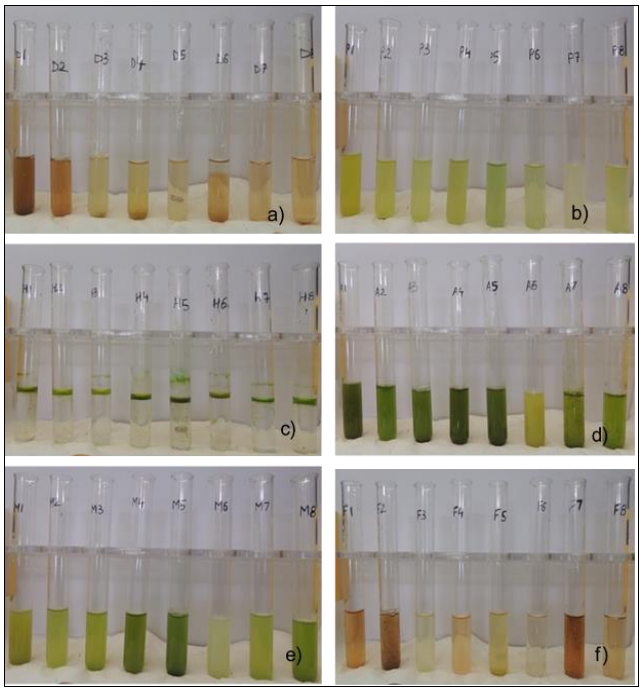

Fig 5: Green synthesised Zinc nanoparticles of *Chromolaena odorata*, *Justicia adhatoda*, *Glycosmis pentaphylla*, *Azadirachta indica*, *Gliricidia sepium*, *Piper nigrum*, *Ocimum tenuiflorum* and *Tabernaemontana divaricate* a) dry leaf distilled water extracts, b) dry leaf propane extracts, c) dry leaf hexane extracts, d) dry leaf acetone extracts, e) dry leaf methanol extracts, f) fresh leaf distilled water extracts.

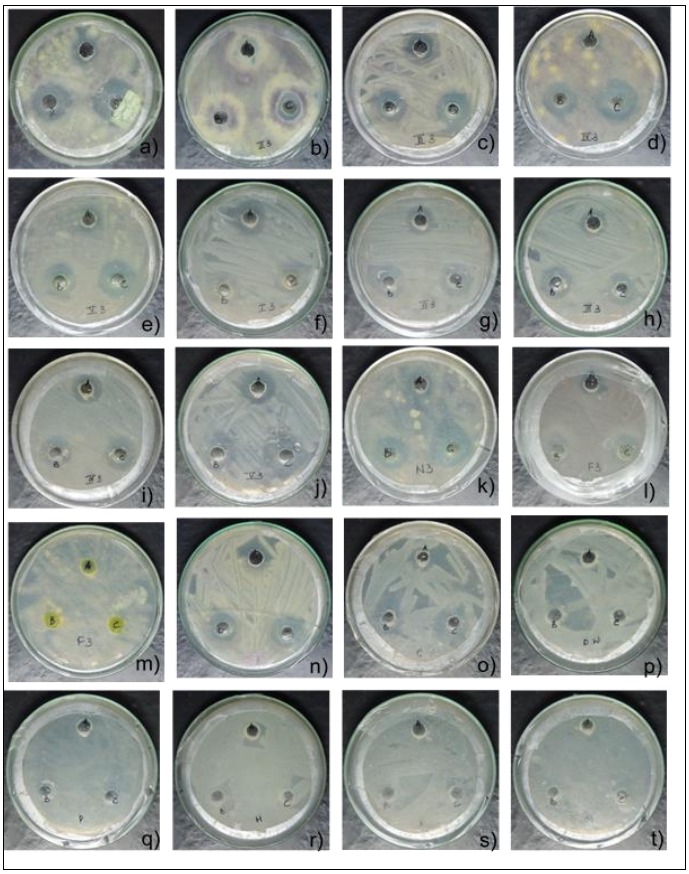

Fig 6: Antifungal activity study using well diffusion method of Pannal (*Glycosmis pentaphylla*) 24 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e) Methanol, f) to j) above mentioned test plate in same order for Zn nanoparticles, k) fresh extract nanoparticle (Cu), l) fresh extract nanoparticle (Zn), m) fresh extract alone, n) Cu control plate, o) Zn control plate, p) to t) solvent control plates (Distilled water, Propane, Hexane, Acetone, Methanol).

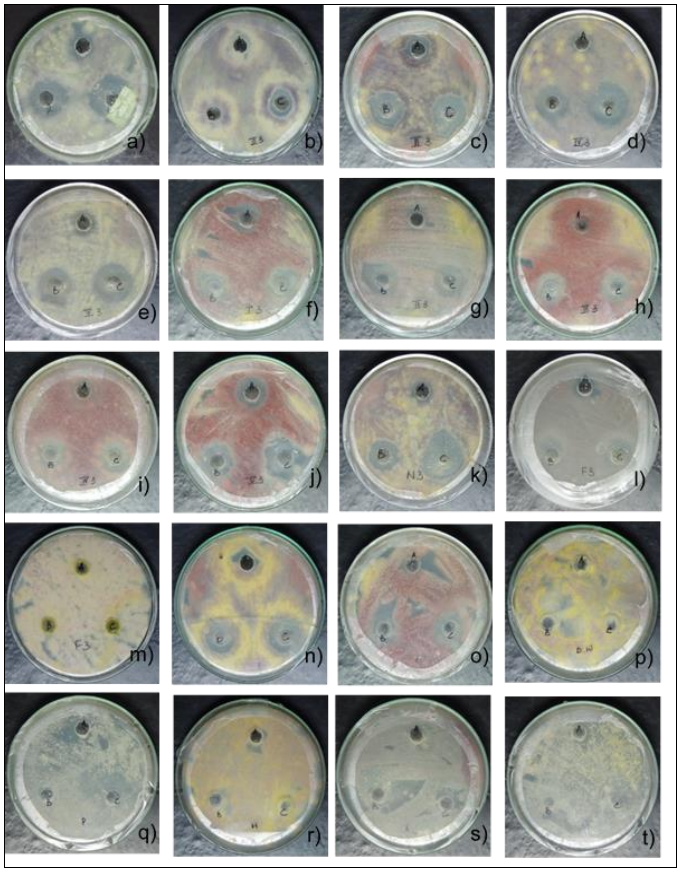

Fig 7: Antifungal activity study using well diffusion method of Pannal (*Glycosmis pentaphylla*) 48 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e) Methanol, f) to j) above mentioned test plate in same order for Zn nanoparticles, k) fresh extract nanoparticle (Cu), l) fresh extract nanoparticle (Zn), m) fresh extract alone, n) Cu control plate, o) Zn control plate, p) to t) solvent control plates (Distilled water, Propane, Hexane, Acetone, Methanol).

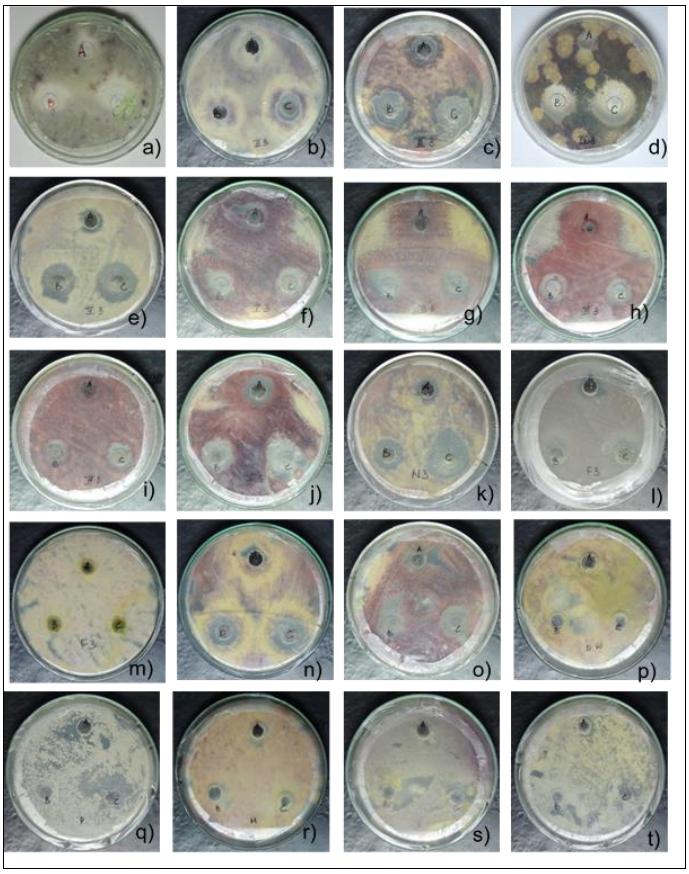

Fig 8: Antifungal activity study using well diffusion method of Pannal (*Glycosmis pentaphylla*) 72 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e) Methanol, f) to j) above mentioned test plate in same order for Zn nanoparticles, k) fresh extract nanoparticle (Cu), l) fresh extract nanoparticle (Zn), m) fresh extract alone, n) Cu control plate, o) Zn control plate, p) to t) solvent control plates (Distilled water, Propane, Hexane, Acetone, Methanol).

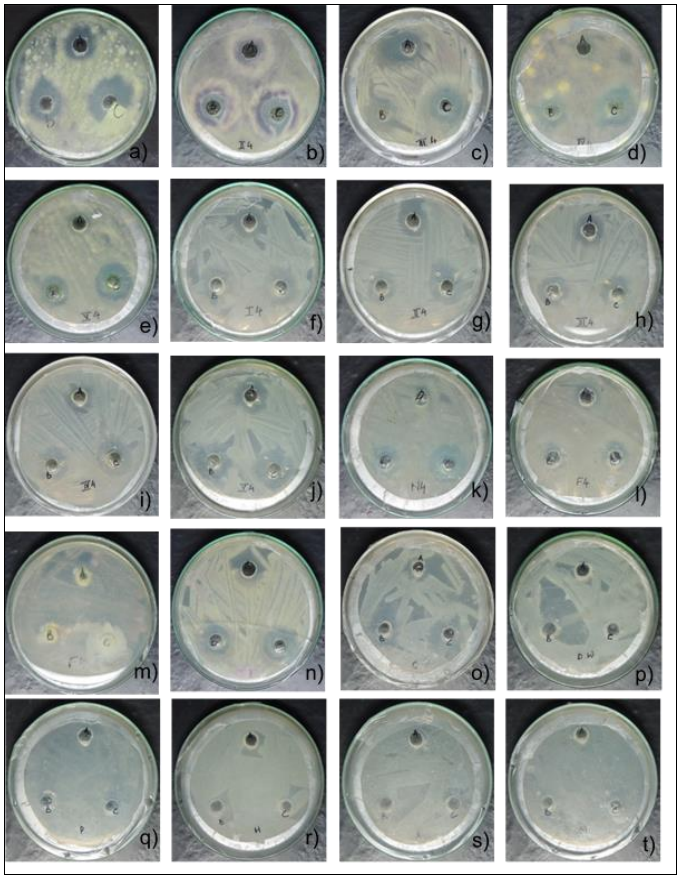

Fig 9: Antifungal activity study using well diffusion method of Neem (*Azadirachta indica*) 24 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e) Methanol, f) to j) above mentioned test plate in same order for Zn nanoparticles, k) fresh extract nanoparticle (Cu), l) fresh extract nanoparticle (Zn), m) fresh extract alone, n) Cu control plate, o) Zn control plate, p) to t) solvent control plates (Distilled water, Propane, Hexane, Acetone, Methanol).

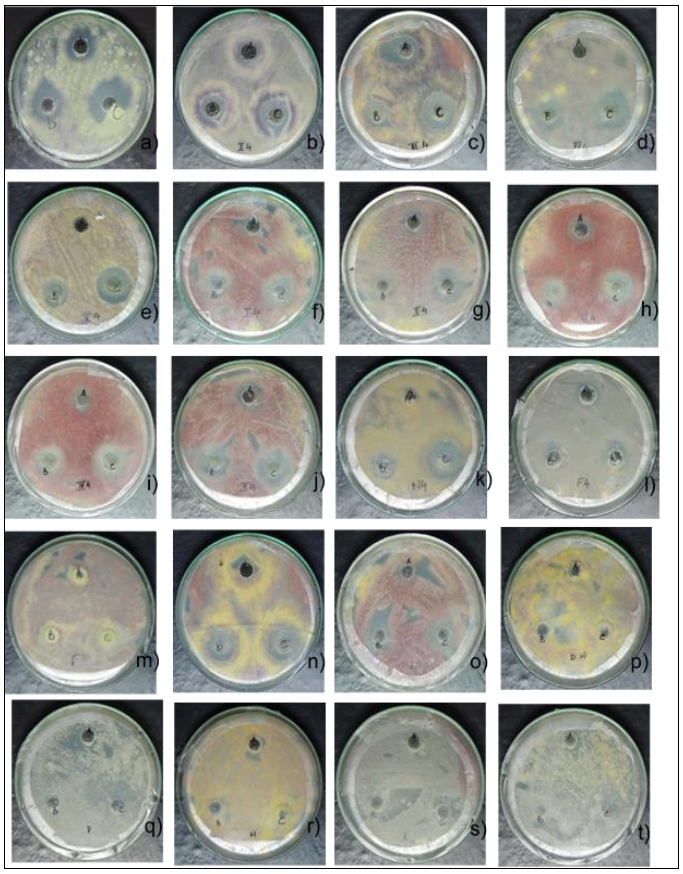

Fig 10: Antifungal activity study using well diffusion method of Neem (*Azadirachta indica*) 48 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e) Methanol, f) to j) above mentioned test plate in same order for Zn nanoparticles, k) fresh extract nanoparticle (Cu), l) fresh extract nanoparticle (Zn), m) fresh extract alone, n) Cu control plate, o) Zn control plate, p) to t) solvent control plates (Distilled water, Propane, Hexane, Acetone, Methanol)

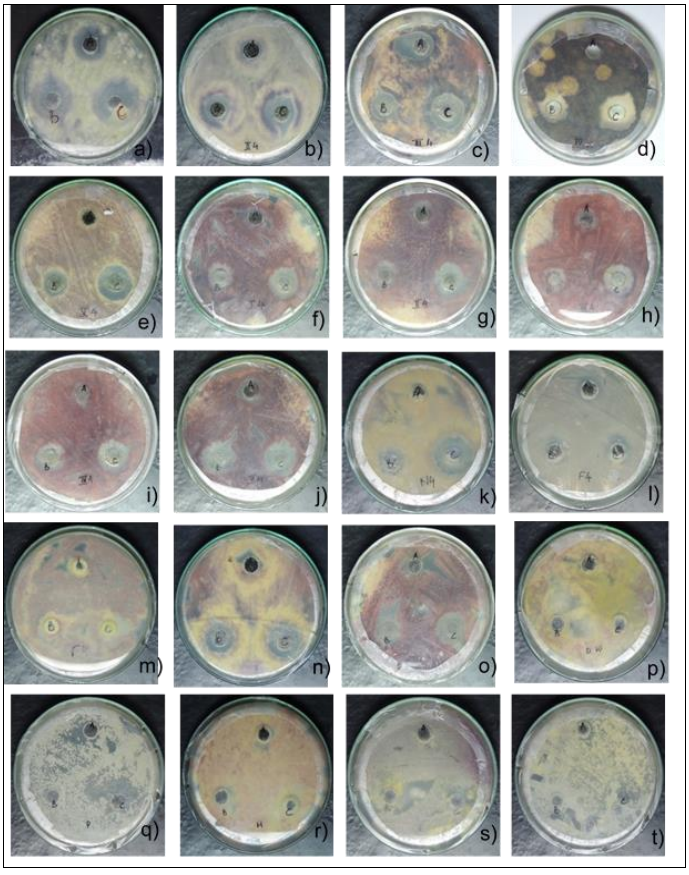

Fig 11: Antifungal activity study using well diffusion method of Neem (*Azadirachta indica*) 72 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e) Methanol, f) to j) above mentioned test plate in same order for Zn nanoparticles, k) fresh extract nanoparticle (Cu), l) fresh extract nanoparticle (Zn), m) fresh extract alone, n) Cu control plate, o) Zn control plate, p) to t) solvent control plates (Distilled water, Propane, Hexane, Acetone, Methanol).

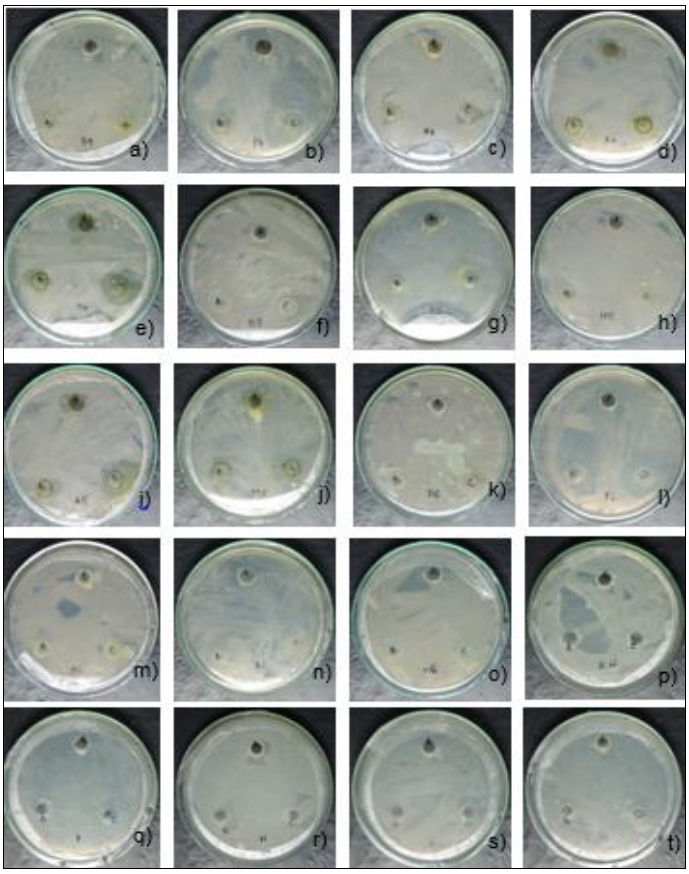

Fig 12: Antifungal activity study using well diffusion method of Neem, (*Azadirachta indica*) 24 hrs leaf dry extract a) control plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e) Methanol, f) to j) above mentioned control plate in same order for Cheema Konna (*Gliricidia sepium*), k) to o) above mentioned control plate in same order for Pepper (*Piper nigrum*), p) to t) solvent control plates (Distilled water, Propane, Hexane, Acetone, Methanol).

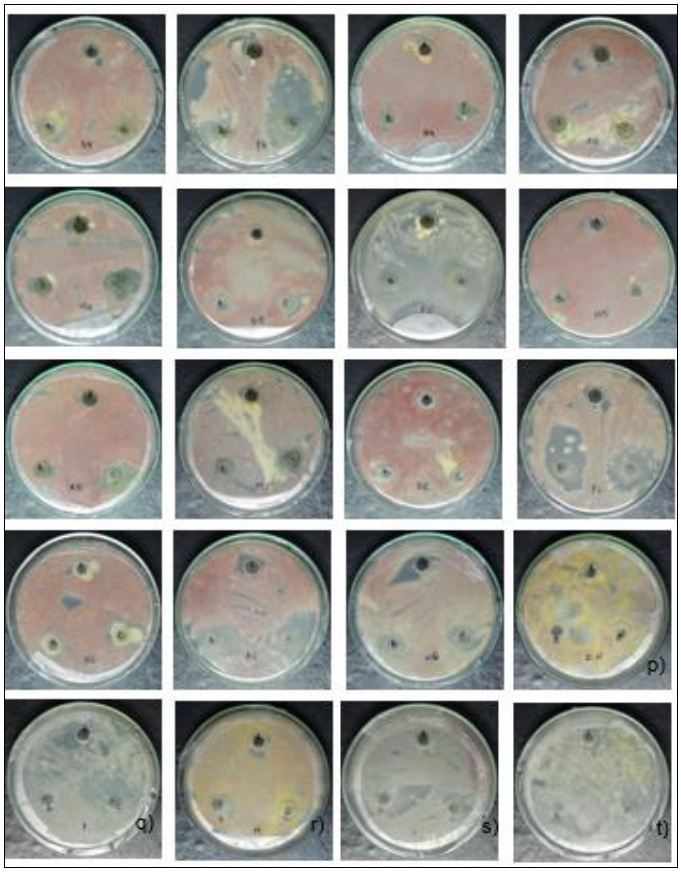

Fig 13: Antifungal activity study using well diffusion method of Neem, (*Azadirachta indica*) 48 hrs leaf dry extract a) control plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e) Methanol, f) to j) above mentioned control plate in same order for Cheema Konna (*Gliricidia sepium*), k) to o) above mentioned control plate in same order for Pepper (*Piper nigrum*), p) to t) solvent control plates (Distilled water, Propane, Hexane, Acetone, Methanol).

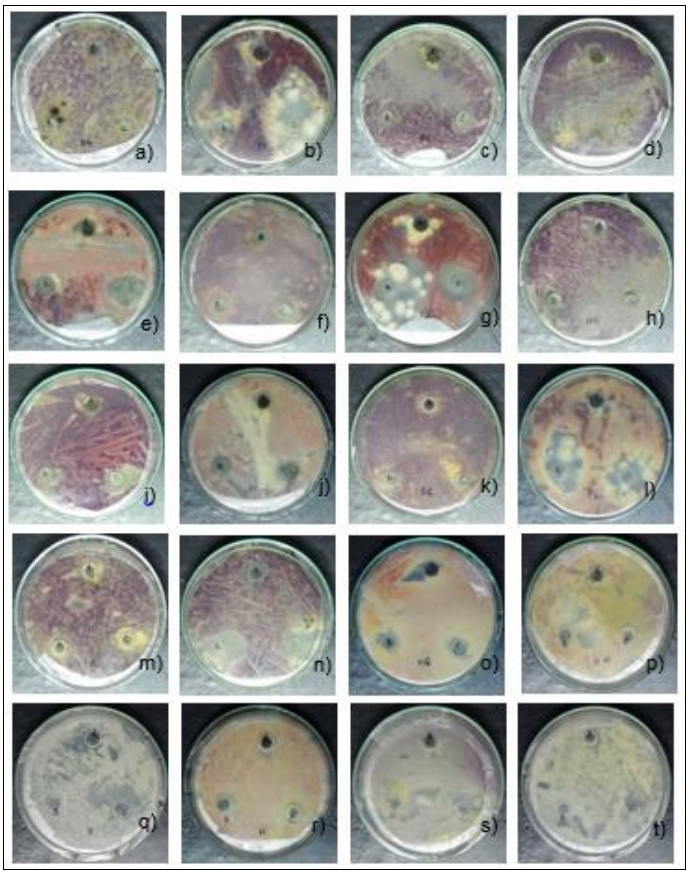

Fig 14: Antifungal activity study using well diffusion method of Neem, (*Azadirachta indica*) 72 hrs. leaf dry extract a) control plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e) Methanol, f) to j) above mentioned control plate in same order for Cheema Konna (*Gliricidia sepium*), k) to o) above mentioned control plate in same order for Pepper (*Piper nigrum*), p) to t) solvent control plates (Distilled water, Propane, Hexane, Acetone, Methanol).

Table 3: Zone of inhibition (24 hrs) against *Fusarium oxysporum* cubense by the Distilled Water extract (50, 100 and 150 µl) of various plant leaves (*Glycosmis pentaphylla, Azadirachta indica*). Control; $CuSO_4$ and $ZnSO_4$ solution, Sample; dry leaf extract in distilled water.

Plant name	Nanoparticles	Control			Sample			Test		
		Measure of zone of inhibition (cm)			Measure of zone of inhibition (cm)			Measure of zone of inhibition (cm)		
		50	100	150	50	100	150	50	100	150
Pannal (distilled water)	Copper	1.7	2.5	3	-	-	-	1.5	2.8	3.1
	Zinc	1.4	1.5	2	-	-	-	2	2.3	2.5
Neem (distilled water)	Copper	1.7	2.5	3	-	-	-	2.2	2.5	3.5
	Zinc	1.4	1.5	2	-	-	-	2	2.4	2.5
Pannal (proponic extract)	Copper	1.7	2.5	3	1.8	2	2.5	2.5	2.8	3
	Zinc	1.4	1.5	2	1.8	2	2.5	2	2.6	2.7
Neem (proponic extract)	Copper	1.7	2.5	3	1.5	1.7	2.4	2.5	2.8	3
	Zinc	1.4	1.5	2	1.5	1.7	2.4	2	2	2.4
Pannal (hexonic extract)	Copper	1.7	2.5	3	-	-	-	1.8	2.3	2.5
	Zinc	1.4	1.5	2	-	-	-	1.7	2.1	2.4
Neem (hexonic extract)	Copper	1.7	2.5	3	-	-	1.7	1.7	1.8	2.3
	Zinc	1.4	1.5	2	-	-	1.7	1.7	2.1	2.2
Pannal (acetonic extract)	Copper	1.7	2.5	3	1.6	1.6	1.8	1.4	2.2	3.2
	Zinc	1.4	1.5	2	1.6	1.6	1.8	2	2.2	2.3
Neem (acetonic extract)	Copper	1.7	2.5	3	2	2	2	1.3	2.3	3
	Zinc	1.4	1.5	2	2	2	2	1.7	2.2	2.6
Pannal (methanonic extract)	Copper	1.7	2.5	3	2.1	2.2	2.5	1.6	2.2	2.4
	Zinc	1.4	1.5	2	2.1	2.2	2.5	2.1	2.2	2.5
Neem (methanonic extract)	Copper	1.7	2.5	3	2.1	2.4	2.5	1.8	2	2.5
	Zinc	1.4	1.5	2	2.1	2.4	2.5	2.1	2.4	2.5

Table 4: Zone of inhibition (48 hrs.) against *Fusarium oxysporum* cubense by the Distilled Water extract (50, 100 and 150 µl) of various plant leaves (*Glycosmis pentaphylla, Azadirachta indica*). Control; $CuSO_4$ and $ZnSO_4$ solution, Sample; dry leaf extract in distilled water.

Plant name	Nanoparticles	Control			Sample			Test		
		Measure of zone of inhibition (cm)			Measure of zone of inhibition (cm)			Measure of zone of inhibition (cm)		
		50	100	150	50	100	150	50	100	150
Pannal (distilled water)	Copper	1.6	2.1	2.7	-	-	-	1.5	2.6	2.9
	Zinc	1.3	1.4	1.8	-	-	-	1.9	2	2.1
Neem (distilled water)	Copper	1.6	2.1	2.7	-	-	-	2.2	2.7	3.2
	Zinc	1.3	1.4	1.8	-	-	-	1.7	1.8	2
Pannal (proponic extract)	Copper	1.6	2.1	2.7	2	2.2	3	-	-	1.8
	Zinc	1.3	1.4	1.8	2	2.2	3	1.5	2.1	2.1
Neem (proponic extract)	Copper	1.6	2.1	2.7	1.2	1.5	3	1.5	1.7	2
	Zinc	1.3	1.4	1.8	1.2	1.5	3	1.5	1.6	2
Pannal (hexonic extract)	Copper	1.6	2.1	2.7	-	-	-	1.8	2.2	2.3
	Zinc	1.3	1.4	1.8	-	-	-	1.5	1.9	2
Neem (hexonic extract)	Copper	1.6	2.1	2.7	-	1.5	1.7	2	2	2.3
	Zinc	1.3	1.4	1.8	-	1.5	1.7	1.6	1.7	2.1
Pannal (acetonic extract)	Copper	1.6	2.1	2.7	1.8	1.9	2	1.3	2	3
	Zinc	1.3	1.4	1.8	1.8	1.9	2	1.8	1.8	1.9
Neem (acetonic extract)	Copper	1.6	2.1	2.7	1.5	1.7	-	1.2	2.1	2.9
	Zinc	1.3	1.4	1.8	1.5	1.7	-	1.7	1.9	2
Pannal (methanonic extract)	Copper	1.6	2.1	2.7	1.7	1.9	2.8	1.5	2.3	2.6
	Zinc	1.3	1.4	1.8	1.7	1.9	2.8	2	2.1	2.5
Neem (methanonic extract)	Copper	1.6	2.1	2.7	1.6	1.8	2.5	-	1.7	2.3
	Zinc	1.3	1.4	1.8	1.6	1.8	2.5	1.8	2.2	2.3

Table 5: Zone of inhibition (72 hrs) against *Fusarium oxysporum* cubense by the Distilled Water extract (50, 100 and 150 µl) of various plant leaves (*Glycosmis pentaphylla, Azadirachta indica*). Control; $CuSO_4$ and $ZnSO_4$ solution, Sample; dry leaf extract in distilled water.

Plant name	Nanoparticles	Control			Sample			Test		
		Measure of zone of inhibition (cm)			Measure of zone of inhibition (cm)			Measure of zone of inhibition (cm)		
		50	100	150	50	100	150	50	100	150
PANNAL (Distilled water)	Copper	1.4	2	2.5	-	-	-	1.5	2.5	2.5
	Zinc	1.3	1.5	1.8	-	-	-	1.6	1.8	1.9
NEEM (Distilled water)	Copper	1.4	2	2.5	-	-	-	2.3	2.8	3.3
	Zinc	1.3	1.5	1.8	-	-	-	1.5	1.6	1.9
PANNAL (Proponic extract)	Copper	1.4	2	2.5	-	1.5	2.1	1.4	1.1	1.6
	Zinc	1.3	1.5	1.8	-	1.5	2.1	-	1.8	1.9
NEEM (Proponic extract)	Copper	1.4	2	2.5	-	1.4	2	1.4	1.6	1.8
	Zinc	1.3	1.5	1.8	-	1.4	2	1.5	1.6	2.1
PANNAL (Hexonic extract)	Copper	1.4	2	2.5	-	-	-	1.7	2.1	2.2
	Zinc	1.3	1.5	1.8	-	-	-	-	1.7	1.8
NEEM (Hexonic extract)	Copper	1.4	2	2.5	-	1.3	1.5	1.7	1.7	2.3
	Zinc	1.3	1.5	1.8	-	1.3	1.5	1.5	1.6	2
PANNAL (Acetonic extract)	Copper	1.4	2	2.5	1.6	1.7	1.5	-	2	2.9
	Zinc	1.3	1.5	1.8	1.6	1.7	1.5	1.3	1.8	1.9
NEEM (Acetonic extract)	Copper	1.4	2	2.5	1.3	1.5	-	-	1.4	2.1
	Zinc	1.3	1.5	1.8	1.3	1.5	-	1.5	1.7	2
PANNAL (Methanonic extract)	Copper	1.4	2	2.5	1.7	1.4	2.4	1.6	2.9	2.6
	Zinc	1.3	1.5	1.8	1.7	1.4	2.4	1.9	2.2	2.5
NEEM (Methanonic extract)	Copper	1.4	2	2.5	1.6	1.8	2.5	-	1.5	2.3
	Zinc	1.3	1.5	1.8	1.6	1.8	2.5	1.6	2	2

Table 6: Zone of inhibition against *Fusarium oxysporum* cubense by the various solvents (Distilled water, Propanol, Hexane, Acetone, Methanol) in 50, 100 and 150 µl volume during 24, 48 and 72 hrs of incubation period.

No	Control solvents	Measure of zone of inhibition (cm), 24 hrs.			Measure of zone of inhibition (cm), 48 hrs.			Measure of zone of inhibition (cm), 72 hrs.		
		50	100	150	50	100	150	50	100	150
1	Distilled water	-	-	-	-	-	-	-	-	-
2	Propanol	1.7	-	2.1	1.3	-	2	-	-	1.5
3	Hexane	1.6	-	-	-	-	-	-	-	-
4	Acetone	-	-	1.7	-	-	1.5	-	-	1.4
5	Methanol	1.4	1.5	1.7	-	-	1.5	-	-	1.3

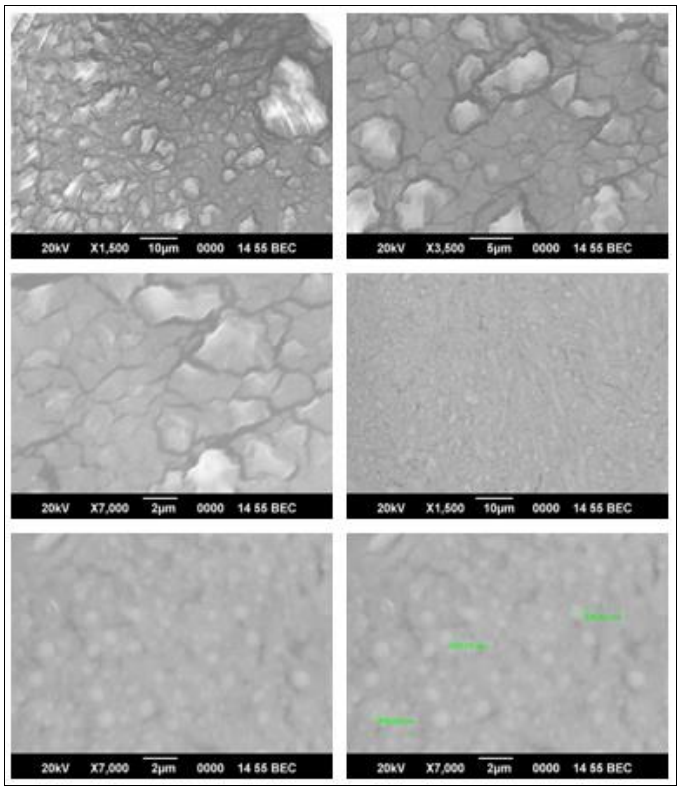

Fig 15: Zinc nanoparticle formation of Neem (*Azadirachta indica*) dry leaf methanol extract under SEM imaging system with various resolutions.

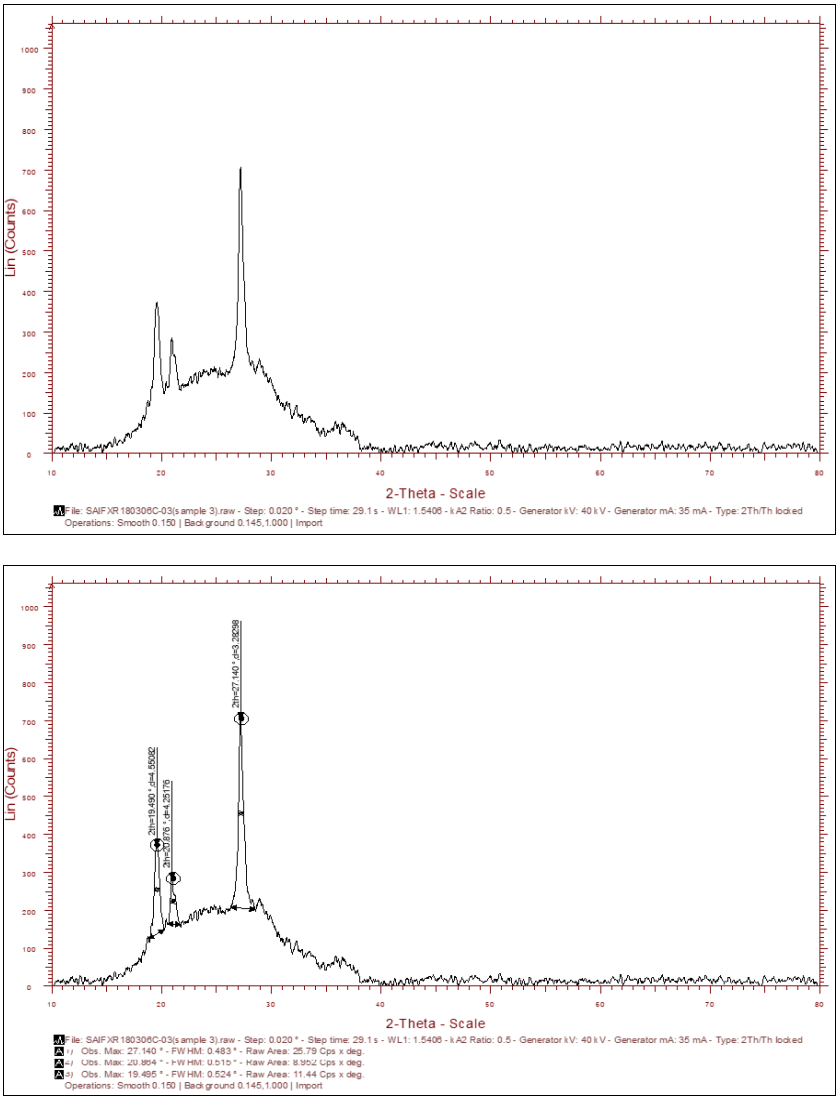

Fig 16: Zinc nanoparticle formation of Neem (*Azadirachta indica*) dry leaf methanol extract under XRD imaging system.

Table 7: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Distilled water extract of Pannal (*Glycosmis pentaphylla*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr.	1.060	0.100	0.455	0.043	0.273	0.031
1 hr.	1.099	0.156	0.474	0.078	0.294	0.047
1 ½ hr.	1.138	0.169	0.512	0.080	0.322	0.069
2 hr.	1.152	0.150	0.537	0.069	0.351	0.083
2 ½ hr.	1.168	0.130	0.586	0.062	0.385	0.075

Table 8: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Distilled water extract of Neem (*Azadirachta indica*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr.	1.184	0.070	0.702	0.042	0.712	0.035
1 hr.	1.203	0.095	0.814	0.061	0.736	0.049
1 ½ hr.	1.241	0.108	0.886	0.083	0.783	0.074
2 hr.	1.289	0.083	0.935	0.074	0.799	0.083
2 ½ hr.	1.315	0.074	0.948	0.080	0.826	0.078

Table 9: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Propane extract of Pannal (*Glycosmis pentaphylla*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr.	1.001	0.024	0.620	0.017	0.543	0.011
1 hr.	1.153	0.051	0.664	0.037	0.567	0.026
1 ½ hr.	1.167	0.076	0.691	0.055	0.584	0.043
2 hr.	1.189	0.062	0.715	0.063	0.609	0.068
2 ½ hr.	2..001	0.053	0.734	0.068	0.626	0.061

Table 10: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Propane extract of Neem (*Azadirachta indica*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr.	1.044	0.124	0.752	0.109	0.722	0.114
1 hr.	1.080	0.142	0.824	0.127	0.743	0.137
1 ½ hr.	1.091	0.167	0.861	0.146	0.771	0.158
2 hr.	1.118	0.158	0.916	0.150	0.796	0.149
2 ½ hr.	1.130	0.149	0.954	0.155	0.824	0.142

Table 11: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Hexane extract of Pannal (*Glycosmis pentaphylla*) leaves during different time of incubation

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr.	0.108	0.109	0.044	0.084	0.020	0.098
1 hr.	0.124	0.137	0.051	0.091	0.025	0.125
1 ½ hr.	0.131	0.158	0.063	0.126	0.031	0.171
2 hr.	0.150	0.172	0.069	0.133	0.039	0.182
2 ½ hr.	0.157	0.175	0.071	0.130	0.045	0.179

Table 12: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Hexane extract of Neem (*Azadirachta indica*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr.	0.064	0.421	0.027	0.512	0.009	0.302
1 hr.	0.071	0.486	0.034	0.549	0.015	0.327
1 ½ hr.	0.079	0.493	0.039	0.596	0.028	0.394
2 hr.	0.083	0.506	0.046	0.617	0.030	0.414
2 ½ hr.	0.087	0.518	0.058	0.587	0.034	0.456

Table 13: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Acetone extract of Pannal (*Glycosmis pentaphylla*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.007	0.082	1.032	0.076	1.041	0.094
1 hr	1.048	0.154	1.057	0.084	1.068	0.106
1 ½ hr	1.079	0.248	1.080	0.092	1.094	0.124
2 hr	1.123	0.261	1.086	0.137	1.131	0.133
2 ½ hr	1.157	0.285	1.104	0.141	1.307	0.145

Table 14: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Acetone extract of Neem (*Azadirachta indica*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.453	0.429	1.502	0.507	1.630	0.618
1 hr	1.597	0.467	1.536	0.542	1.654	0.634
1 ½ hr	1.618	0.499	1.597	0.559	1.702	0.686
2 hr	1.649	0.527	1.625	0.551	1.775	0.672
2 ½ hr	1.706	0.529	1.649	0.547	1.903	0.644

Table 15: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Methanol extract of Pannal (*Glycosmis pentaphylla*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	0.910	0.842	0.924	0.853	0.958	0.872
1 hr	0.924	0.862	0.937	0.876	0.973	0.888
1 ½ hr	0.943	0.890	0.958	0.881	0.991	0.921
2 hr	0.978	0.917	0.981	0.917	1.017	0.938
2 ½ hr	1.006	0.922	1.011	0.936	1.032	0.945

Table 16: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Methanol extract of Neem (*Azadirachta indica*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.014	0.946	1.039	0.872	1.042	0.924
1 hr	1.064	0.961	1.067	0.894	1.059	0.937
1 ½ hr	1.123	0.938	1.092	0.904	1.097	0.946
2 hr	1.147	0.921	1.159	0.967	1.124	0.973
2 ½ hr	1.160	0.905	1.186	0.955	1.167	0.942

Table 17: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Fresh extract of Pannal (*Glycosmis pentaphylla*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	0.047	0.016	0.057	0.042	0.050	0.027
1 hr	0.051	0.026	0.062	0.067	0.064	0.038
1 ½ hr	0.067	0.030	0.071	0.082	0.069	0.049
2 hr	0.072	0.045	0.083	0.076	0.081	0.057
2 ½ hr	0.083	0.052	0.096	0.066	0.094	0.067

Table 18: Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Fresh extract of Neem (*Azadirachta indica*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	0.057	0.034	0.064	0.021	0.060	0.025
1 hr	0.064	0.051	0.081	0.029	0.074	0.037
1 ½ hr	0.082	0.042	0.094	0.034	0.079	0.049
2 hr	0.091	0.029	0.105	0.038	0.085	0.050
2 ½ hr	0.110	0.026	0.109	0.042	0.093	0.041

5. Results and Discussion

5.1 Synthesis of nanoparticles

Nanoparticles were synthesized from the leaf extracts of *Glycosmis pentaphylla*, *Azadirachta indica*, using solvents like distilled water, propane, hexane, methanol and acetone.

5.1.1 Copper nanoparticles

Copper nanoparticles were synthesized from leaf extract of different plants (*Glycosmis pentaphylla*, *Azadirachta indica*). Leaf extract was added to 100Mm copper sulphate solution and kept to reaction to take place. A colour change was observed from blue to pale yellow. This occurred due to the reduction of copper ions present in the solution

5.1.2 Zinc nanoparticles

Zinc nanoparticles were synthesized from leaf extract of different plants (*Glycosmis pentaphylla*, *Azadirachta indica*). Leaf extract was added to 100Mm zinc sulphate solution and kept to reaction to take place. A colour change was observed from colourless to pale brown. This occurred due to the reduction of zinc ions present in the solution.

5.2 Characterization of nanoparticles

5.2.1 Copper nanoparticles-UV spectroscopy

Synthesized copper nanoparticles were characterized by UV-VIS spectrophotometry. The maximum peak was found to be at 435 nm for the leaf extracts of, *Glycosmis pentaphylla*, *Azadirachta indica* with distilled water and propane and 680nm with hexane, acetone and methanol solvent extracts. The intensity of the peak was increased with time until the reduction completes.

5.2.2 Zinc nanoparticles-UV spectroscopy

Synthesized zinc nanoparticles were characterized by UV-VIS spectrophotometry. The maximum peak was found to be at 385nm for the leaf extracts *of Glycosmis pentaphylla*, *Azadirachta indica* with each solvent.

The SEM-XRD analysis proved the effective formation of copper and zinc nanoparticles in all the samples.

5.3 Antifungal assay

The leaf extracts of *Glycosmis pentaphylla*, *Azadirachta indica* with solvents distilled water, propane, hexane, methanol and acetone and the fresh extract showed the growth inhibitory effects against *Fusarium oxysporum cubense*.

5.3.1 Pannal (*Glycosmis pentaphylla*)

With the distilled water, propane and acetone extracts the anti-fungal activity was more with both copper and zinc nanoparticles. Propane was the best solvent for this plant. The fresh extract showed the highest inhibitory action against *Fusarium oxysporum*.

5.3.2 Neem (*Azadirachta indica*)

Copper and zinc nanoparticles with all the solvents except hexane showed a higher zone of inhibition and its result remained almost constant for 48 hours with slight differences.

6. Conclusions

The results shows that leaf extracts of *Glycosmis pentaphylla*, *Azadirachta indica*, with solvents distilled water, propane, hexane, methanol and acetone and the fresh extract are used for the synthesis of copper and zinc nanoparticles. Copper and zinc nanoparticle shows greater antifungal activity than copper sulphate and zinc sulphate, respectively and leaf extract. The maximum zone of inhibition was at 150 µl for all the bacterial cultures. It indicates that the zone of inhibition increases with as the concentration of nanoparticles increased. An overall result showed Distilled water, Methanol and Propane as a good solvents and Tulsi as one of the best remedy against the Panama wilt disease.

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