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## Green Synthesis of Copper and Zinc Nanoparticles from *Gliricidia sepium* and *Piper nigrum* and Evaluation of their Antifungal Activity against *Fusarium oxysporum cubense*: An Overview

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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 *Gliricidia sepium* and *Piper nigrum*. 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 150 $\mu$ l 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.

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### 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). Nano-technology mainly consists of the processing of, separation, consolidation, and deformation

of materials by one atom or one molecule (Prasad *et al.*, 2008). Nanoparticles has 1-100 nm in size and they possess novel physical and chemical properties (Sajeshkumar *et al.*, 2015a; Sajeshkumar *et al.*, 2015b). Nanoparticles bear antibacterial properties (Hajipour *et al.*, 2012). 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). 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 (Ramya Devi *et al.*, 2012).

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). 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).

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). 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) 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).

### 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).

### **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). 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).

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). 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).

### **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). 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.

### **Zinc nanoparticles**

Zinc nanoparticles have wide application; various synthetic methods have been employed to produce ZnNps (Chen *et al.*, 2007). Zinc nanoparticles can produced from zinc oxide and zinc sulphate. Zinc nanoparticles has several medicinal uses, which harm skin, stomach, intestine and lymphatic 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).

### **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). 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.

### **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 in 1958 and the subsequent introduction of IV AmB in 1960. Antifungal creams, liquids and sprays are used to treat fungal infections.

## 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.

## Objectives

Synthesis of copper and zinc nanoparticles using leaf extracts of Cheema konna (*Gliricidia sepium*) and Black pepper (*Piper nigrum*) and determine the antifungal properties of these nanoparticles against *Fusarium oxysporum cubense*.

## 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.

## 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).

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).

### *Gliricidia sepium* (Cheema konna)

*Gliricidia sepium* is a medium-sized leguminous tree and is used in places like the Caribbean, the Philippines, India, Sri Lanka and West Africa. It is a thornless tree which usually attains a height of 10-12 m and its ranching is frequently from the base with basal diameters reaching 50-70 cm. The bark is smooth but can vary in colour from whitish grey to deep red brown. It is majorly found in Central American countries and Mexico. It is native only in the seasonally dry forest and in areas where sufficient moisture prevails, the tree does not become leafless.

*Gliricidia sepium* in cultivation in wet areas may often flower, although sets little if any fruit. It is used for many other purposes including live fencing, fodder, firewood, green manure, intercropping, and rat poison. They are used for intercropping in part because they fix nitrogen in the soil and tolerate low soil fertility, so when they are interplanted with crops they can boost crop yields significantly, without the need of chemical fertilizers. (Simons and Stewart, 2007)

### Taxonomical classification (*Gliricidia sepium*; *Cheema konna*)

Kingdom: Plantae-- planta, plantes, plants, vegetal

Subkingdom: Viridiplantae

Superdivision: Embryophyta

Division: Tracheophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: *Gliricidia*

Species: *Gliricidia sepium*

### *Piper nigrum* (Black pepper)

Spices are consumed daily and used to enhance the flavor and aroma of food. Black pepper is one such spice which is used commonly all over the world. It is a woody climber and grown mainly in South Western parts of India and countries like Indonesia, Malaysia, Sri Lanka etc. (Ravindran, 2005) Black pepper is an important spice, appreciated for both its aroma and its pungency. It is analyzed that black pepper had digestive power, improves appetite, cures cold, cough, diseases of the throat, intermittent fever, colic, dysentery, worms and piles.

It also possesses a broad-spectrum of antimicrobial activity and gave favourable results in terms of mutagenic and carcinogenic properties.

There antioxidant properties have been useful as food additives to provide protection against oxidative degradation of foods and also reduce lipid peroxidation.

Piper-amides extracted has shown insecticidal activities and even caryophyllene extracted from *P. nigrum* showed anesthetic activity. (Gulcin, 2005)

### **Taxonomical classification (*Piper nigrum*; Black pepper)**

Kingdom: Plantae-- planta, plantes, plants, vegetal

Subkingdom: Viridiplantae

Superdivision: Embryophyta

Division: Tracheophyta

Class: Magnoliopsida

Order: Piperales

Family: Piperaceae

Genus: Piper

Species: *Piper nigrum*

### **Hypothesis**

The current research work is based on the following hypothesis

Plant extracts of Cheema konna (*Gliricidia sepium*) and Black pepper (*Piper nigrum*) could be used as antifungal agents.

These plant extracts could be used in formulating nanoparticles (copper and zinc) and their antifungal activity vary widely.

### **Materials and Methods**

#### **Study area**

Kerala state covers an area of 38,863 km<sup>2</sup> with a population density of 859 per km<sup>2</sup> 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).

Maximum rainfall occurs from June to September mainly due to South West Monsoon and temperatures are highest in May and November.

### **Sample collection**

Samples of Cheema konna (*Gliricidia sepium*) and Black pepper (*Piper nigrum*) 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°C for 48hrs and later stored in air tight polyethylene zipper bag for analysis.

### **Extraction method**

#### **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.

#### **Fresh extraction**

Leaf extract is prepared with 10 g of fresh leaves (*Gliricidia sepium* and *Piper nigrum*) thoroughly washed with tap water and then with DH<sub>2</sub>O and cut into small pieces.

It is then crushed in a pistil and motor by adding 30 ml of DH<sub>2</sub>O. It is then filtered using a filter paper into a container and is then stored at 4°C for further use.

### **Synthesis of nanoparticles**

#### **Copper nanoparticles**

The stock solution is prepared by dissolving 2.49 g of Copper sulphate (CuSO<sub>4</sub>) in 100 ml of DH<sub>2</sub>O. Add 9ml of the 100mM CuSO<sub>4</sub> 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.

#### **Zinc nanoparticles**

The stock solution is prepared by dissolving 2.87g of Zinc sulphate (ZnSO<sub>4</sub>) in 100ml of DH<sub>2</sub>O. Add 9ml of the 100mM ZnSO<sub>4</sub> 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.

## Test microorganisms

*Fusarium 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.

## Solvents

### 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.

### Propane

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

### Hexane

It is an alkane of six carbon atoms, with the chemical formula  $C_6H_{14}$ . They are colorless liquids, odorless 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. It is highly flammable and its vapours can be explosive.

### Acetone

It is an organic compound with the formula  $(CH_3)_2CO$ . It is a colorless, 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.

### Methanol

It is the simplest alcohol, consisting of a methyl group linked to a hydroxyl group. It is a light, volatile, colorless, 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.

## Characterization of nanoparticles

### 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.

### 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.

### 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  $\mu l$  of nanoparticle solution, control (stock solution) and sample (fresh and dry leaf extracts). The plates were incubated at  $25^\circ C$  for 72 hours after which the diameter of zones of inhibition were measured on regular intervals (24, 48 and 72 hrs).

**Table.1** Different vernacular names of Cheema konna (*Gliricidia sepium*), around the globe and India.

Language	Names
<b>Scientific names</b>	<i>Gliricidia sepium</i>
<b>Name in various global languages</b>	
<b>French</b>	
<b>German</b>	
<b>English</b>	Mexican lilac
<b>Name in various Indian languages</b>	
<b>Sanskrit</b>	
<b>Hindi</b>	
<b>Urdu</b>	
<b>Bengali</b>	Saranga
<b>Kannada</b>	Gobbarda mara
<b>Telugu</b>	Madri
<b>Malayalam</b>	Seema konna
<b>Tamil</b>	Seemai agathi

**Table.2** Different vernacular names of Black pepper (*Piper nigrum*L.) around the globe and India.

Language	Names
<b>Scientific names</b>	<i>Piper nigrum</i> L.
<b>Name in various global languages</b>	
<b>French</b>	Poivre
<b>German</b>	Pfeffer
<b>English</b>	Black pepper
<b>Name in various Indian languages</b>	
<b>Sanskrit</b>	Marich
<b>Hindi</b>	Kali mirch
<b>Urdu</b>	Syah mirch
<b>Marathi</b>	Golmirich
<b>Kannada</b>	Karimenasu
<b>Gujarati</b>	Kalomirich
<b>Malayalam</b>	Kurumulaku
<b>Tamil</b>	Kurumilagu

**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 (*Gliricidia sepium*, *Piper nigrum*). Control; CuSO<sub>4</sub> and ZnSO<sub>4</sub> 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
<b>CHEEMAKONNA</b> (Distilled water)	Copper	1.7	2.5	3	-	-	-	1.9	2.8	3.1
	Zinc	1.4	1.5	2	-	-	-	1.8	2	2.3
<b>PEPPER</b> (Distilled water)	Copper	1.7	2.5	3	-	-	-	3	3	3.5
	Zinc	1.4	1.5	2	-	-	-	1.7	2.3	2.4
<b>CHEEMAKONNA</b> (Proponic extract)	Copper	1.7	2.5	3	1.9	2	2	1.6	2.2	2.5
	Zinc	1.4	1.5	2	1.9	2	2	1.6	2.3	2.6
<b>PEPPER</b> (Proponic extract)	Copper	1.7	2.5	3	2	2.5	3	2.1	2.3	2.5
	Zinc	1.4	1.5	2	2	2.5	3	1.6	2.1	2
<b>CHEEMAKONNA</b> (Hexonic extract)	Copper	1.7	2.5	3	-	1.3	1.4	1.6	2	2.2
	Zinc	1.4	1.5	2	-	1.3	1.4	1.6	2.2	2.5
<b>PEPPER</b> (Hexonic extract)	Copper	1.7	2.5	3	-	-	1.9	2	2	2.5
	Zinc	1.4	1.5	2	-	-	1.9	2.1	2.5	2.5
<b>CHEEMAKONNA</b> (Acetonic extract)	Copper	1.7	2.5	3	1.7	1.5	2	1.4	2	3
	Zinc	1.4	1.5	2	1.7	1.5	2	1.8	2.1	2.4
<b>PEPPER</b> (Acetonic extract)	Copper	1.7	2.5	3	1.7	2	2.2	1.5	2.5	3
	Zinc	1.4	1.5	2	1.7	2	2.2	1.9	2.4	2.3
<b>CHEEMAKONNA</b> (Methanonic extract)	Copper	1.7	2.5	3	2	2.2	2.3	1.9	2	2.6
	Zinc	1.4	1.5	2	2	2.2	2.3	2	2.2	2.3
<b>PEPPER</b> (Methanonic extract)	Copper	1.7	2.5	3	2.1	2.5	2.5	1.5	2.4	2.6
	Zinc	1.4	1.5	2	2.1	2.5	2.5	2.1	2.5	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 (*Gliricidia sepium*, *Piper nigrum*). Control; CuSO<sub>4</sub> and ZnSO<sub>4</sub> 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
<b>CHEEMAKONNA</b> (Distilled water)	Copper	1.6	2.1	2.7	-	-	-	1.7	2.8	3
	Zinc	1.3	1.4	1.8	-	-	-	1.8	1.5	2
<b>PEPPER</b> (Distilled water)	Copper	1.6	2.1	2.7	-	-	-	2.9	2.8	3
	Zinc	1.3	1.4	1.8	-	-	-	1.3	1.8	2
<b>CHEEMAKONNA</b> (Proponic extract)	Copper	1.6	2.1	2.7	1.7	2	2.5	-	-	1.3
	Zinc	1.3	1.4	1.8	1.7	2	2.5	1.3	1.9	2.3
<b>PEPPER</b> (Proponic extract)	Copper	1.6	2.1	2.7	1.8	2.5	3	1.5	1.4	1.9
	Zinc	1.3	1.4	1.8	1.8	2.5	3	1.3	1.8	1.8
<b>CHEEMAKONNA</b> (Hexonic extract)	Copper	1.6	2.1	2.7	-	-	-	1.7	2	2.5
	Zinc	1.3	1.4	1.8	-	-	-	1.4	1.9	2.1
<b>PEPPER</b> (Hexonic extract)	Copper	1.6	2.1	2.7	1.2	1.3	1.6	2	2	2.4
	Zinc	1.3	1.4	1.8	1.2	1.3	1.6	1.3	2	2
<b>CHEEMAKONNA</b> (Acetonic extract)	Copper	1.6	2.1	2.7	1.5	1.6	2.2	1.3	1.8	2.8
	Zinc	1.3	1.4	1.8	1.5	1.6	2.2	-	1.7	1.9
<b>PEPPER</b> (Acetonic extract)	Copper	1.6	2.1	2.7	1.7	2.5	3	1.4	2.4	2.9
	Zinc	1.3	1.4	1.8	1.7	2.5	3	1.5	2.1	2
<b>CHEEMAKONNA</b> (Methanonic extract)	Copper	1.6	2.1	2.7	1.2	1.6	2	1.9	2	2.5
	Zinc	1.3	1.4	1.8	1.2	1.6	2	2.1	2	2
<b>PEPPER</b> (Methanonic extract)	Copper	1.6	2.1	2.7	1.5	1.8	1.9	1.4	2.2	2.6
	Zinc	1.3	1.4	1.8	1.5	1.8	1.9	1.9	2.5	2.4

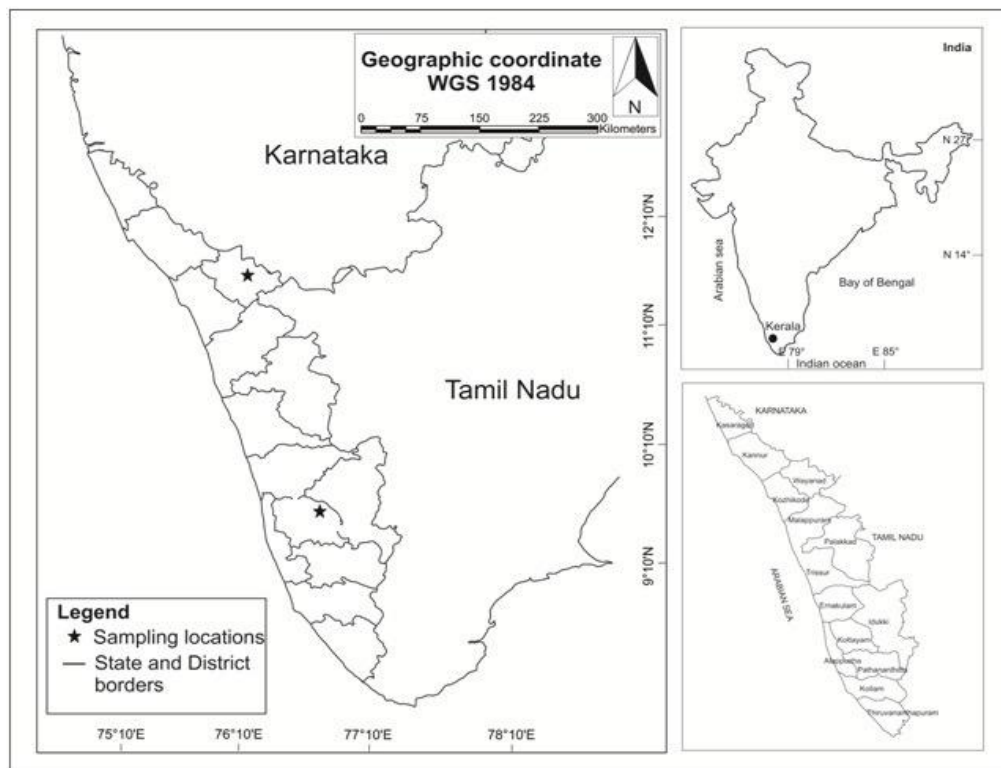
**Table.5** Zone of inhibition (72 hrs) against *Fusarium oxysporum* cubense by the Distilled Water extract (50, 100 and 150 µl) of various plant leaves (*Gliricidia sepium*, *Piper nigrum*). Control; CuSO<sub>4</sub> and ZnSO<sub>4</sub> solution, Sample; dry leaf extract in distilled water.

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
<b>CHEEMAKONNA</b> (Distilled water)	Copper	1.4	2	2.5	-	-	-	1.5	2.8	2.9
	Zinc	1.3	1.5	1.8	-	-	-	1.6	1.8	1.9
<b>PEPPER</b> (Distilled water)	Copper	1.4	2	2.5	-	-	-	2.8	2.6	2.8
	Zinc	1.3	1.5	1.8	-	-	-	-	1.7	2
<b>CHEEMAKONNA</b> (Proponic extract)	Copper	1.4	2	2.5	1.5	2.3	2.5	-	-	1.5
	Zinc	1.3	1.5	1.8	1.5	2.3	2.5	-	1.9	2.1
<b>PEPPER</b> (Proponic extract)	Copper	1.4	2	2.5	1.5	2.4	2.4	1.6	1.4	1.7
	Zinc	1.3	1.5	1.8	1.5	2.4	2.4	-	1.7	1.7
<b>CHEEMAKONNA</b> (Hexonic extract)	Copper	1.4	2	2.5	-	-	-	1.6	2	2.3
	Zinc	1.3	1.5	1.8	-	-	-	-	1.9	2
<b>PEPPER</b> (Hexonic extract)	Copper	1.4	2	2.5	-	1.2	1.4	1.7	2	2.2
	Zinc	1.3	1.5	1.8	-	1.2	1.4	-	1.8	1.9
<b>CHEEMAKONNA</b> (Acetonic extract)	Copper	1.4	2	2.5	1.3	1.5	2	1.2	1.6	2.4
	Zinc	1.3	1.5	1.8	1.3	1.5	2	-	1.5	1.9
<b>PEPPER</b> (Acetonic extract)	Copper	1.4	2	2.5	1.5	2	2.8	-	2	2.5
	Zinc	1.3	1.5	1.8	1.5	2	2.8	-	1.8	1.9
<b>CHEEMAKONNA</b> (Methanonic extract)	Copper	1.4	2	2.5	-	1.4	1.5	1.6	2.1	2.4
	Zinc	1.3	1.5	1.8	-	1.4	1.5	1.7	1.9	1.8
<b>PEPPER</b> (Methanonic extract)	Copper	1.4	2	2.5	1.9	1.5	-	1.7	2.3	2.4
	Zinc	1.3	1.5	1.8	1.9	1.5	-	1.8	2	2.1

**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.

S.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.1** Map of Kerala showing the sample collection point.



**Table.7** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Distilled water extract of Cheemakonna (*Gliricidia sepium*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.412	0.054	0.753	0.049	0.846	0.051
1 hr	1.444	0.063	0.781	0.068	0.867	0.073
1 ½ hr	1.462	0.081	0.798	0.094	0.898	0.094
2 hr	1.497	0.072	0.836	0.115	0.916	0.082
2 ½ hr	1.504	0.070	0.852	0.126	0.928	0.102

**Table.8** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Distilled water extract of Pepper (*Piper nigrum*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.259	0.121	1.055	0.113	0.786	0.103
1 hr	1.321	0.136	1.097	0.124	0.797	0.126
1 ½ hr	1.364	0.167	1.123	0.137	0.820	0.143
2 hr	1.387	0.158	1.146	0.130	0.852	0.138
2 ½ hr	1.402	0.152	1.168	0.119	0.916	0.144

**Table.9** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Propane extract of Cheemakonna (*Gliricidia sepium*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.612	0.032	0.866	0.027	0.914	0.008
1 hr	1.634	0.058	0.884	0.046	0.957	0.024
1 ½ hr	1.655	0.046	0.913	0.073	0.971	0.052
2 hr	1.687	0.033	0.947	0.065	0.993	0.049
2 ½ hr	1.706	0.028	0.963	0.052	1.009	0.041

**Table.10** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Propane extract of Pepper (*Piper nigrum*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.238	0.124	1.055	0.115	0.864	0.097
1 hr	1.267	0.143	1.094	0.131	0.921	0.116
1 ½ hr	1.304	0.138	1.149	0.147	0.957	0.167
2 hr	1.326	0.129	1.167	0.140	0.995	0.152
2 ½ hr	1.336	0.120	1.198	0.137	1.014	0.144

**Table.11** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Hexane extract of Cheemakonna (*Gliricidia sepium*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	0.084	0.239	0.035	0.321	0.007	0.234
1 hr	0.092	0.281	0.041	0.382	0.017	0.265
1 ½ hr	0.099	0.304	0.049	0.403	0.026	0.288
2 hr	0.105	0.338	0.053	0.424	0.035	0.309
2 ½ hr	0.111	0.325	0.061	0.397	0.040	0.328

**Table.12** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Hexane extract of Pepper (*Piper nigrum*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	0.110	0.527	0.120	0.541	0.005	0.552
1 hr	0.118	0.568	0.132	0.567	0.014	0.568
1 ½ hr	0.124	0.591	0.146	0.584	0.024	0.572
2 hr	0.135	0.624	0.159	0.604	0.036	0.579
2 ½ hr	0.138	0.648	0.182	0.629	0.047	0.592

**Table.13** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Acetone extract of Cheemakonna (*Gliricidia sepium*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.034	0.026	1.054	0.035	1.113	0.028
1 hr	1.068	0.048	1.068	0.058	1.134	0.041
1 ½ hr	1.099	0.061	1.089	0.068	1.154	0.058
2 hr	1.132	0.075	1.096	0.105	1.168	0.062
2 ½ hr	1.154	0.084	1.121	0.100	1.185	0.071

**Table.14** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Acetone extract of Pepper (*Piper nigrum*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.125	0.712	1.134	0.729	1.143	0.816
1 hr	1.134	0.734	1.164	0.757	1.157	0.859
1 ½ hr	1.150	0.768	1.172	0.783	1.168	0.877
2 hr	1.168	0.794	1.181	0.808	1.176	0.902
2 ½ hr	1.194	0.826	1.192	0.829	1.191	0.891

**Table.15** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Methanol extract of Cheemakonna (*Gliricidia sepium*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	0.856	0.624	0.864	0.657	0.905	0.582
1 hr	0.904	0.681	0.920	0.671	0.938	0.617
1 ½ hr	0.943	0.699	0.954	0.690	0.961	0.638
2 hr	0.968	0.715	0.993	0.718	0.983	0.655
2 ½ hr	1.108	0.720	1.034	0.729	1.022	0.681

**Table.16** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Methanol extract of Pepper (*Piper nigrum*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	1.032	0.847	1.058	0.859	1.099	0.794
1 hr	1.068	0.869	1.081	0.873	1.126	0.814
1 ½ hr	1.142	0.882	1.093	0.892	1.138	0.837
2 hr	1.167	0.906	1.140	0.905	1.167	0.864
2 ½ hr	1.189	0.921	1.193	0.920	1.194	0.899

**Table.17** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Fresh extract of Cheemakonna (*Gliricidia sepium*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	0.043	0.027	0.051	0.022	0.066	0.019
1 hr	0.049	0.038	0.057	0.029	0.071	0.025
1 ½ hr	0.058	0.047	0.061	0.038	0.076	0.034
2 hr	0.070	0.059	0.068	0.044	0.080	0.039
2 ½ hr	0.072	0.068	0.070	0.046	0.074	0.040

**Table.18** Description of UV absorption spectrum of Copper and Zinc nanoparticles formed from Fresh extract of Pepper (*Piper nigrum*) leaves during different time of incubation.

	Copper	Zinc	Copper	Zinc	Copper	Zinc
Time	435nm	385nm	560nm	435nm	680nm	560nm
½ hr	0.087	0.052	0.094	0.057	0.085	0.045
1 hr	0.094	0.067	0.108	0.062	0.092	0.052
1 ½ hr	0.106	0.072	0.124	0.072	0.114	0.067
2 hr	0.118	0.069	0.146	0.081	0.128	0.075
2 ½ hr	0.124	0.071	0.133	0.088	0.132	0.062

**Fig.2** Cheema konna (*Gliricidia sepium*) description a) tree in natural habitat, b) young leaves, c) mature leaves, d) mature seeds, e) flowers. Photo courtesy: Wikipedia.

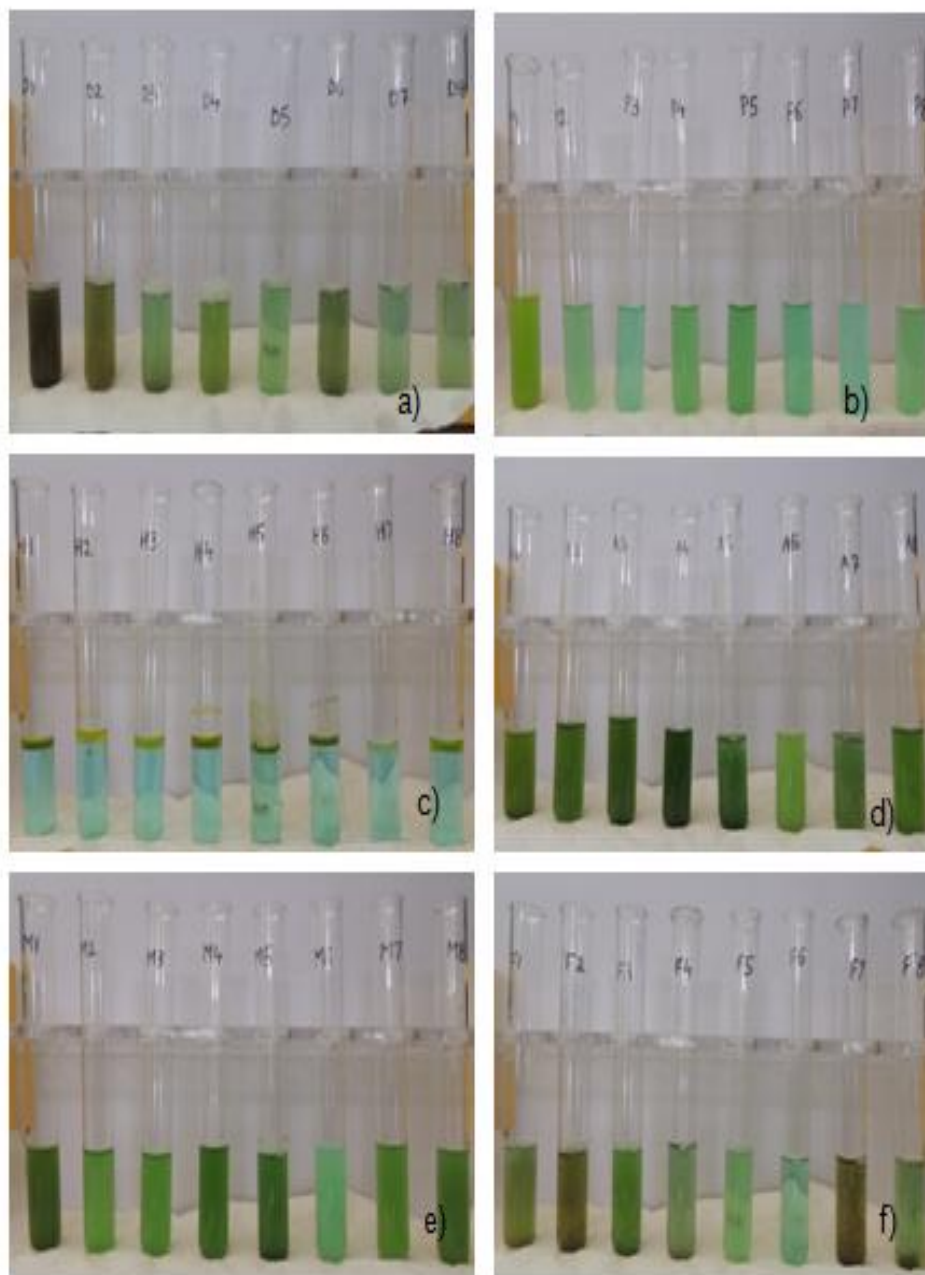


**Fig.3** Black pepper (*Piper nigrum*) description a) fully ripened fruits, b) powdered fruits, c) green mature fruits separated from spike, d) red fully mature fruits separated from spike, e) mature and fully ripened fruits with spike, f) plant with mature spikes.

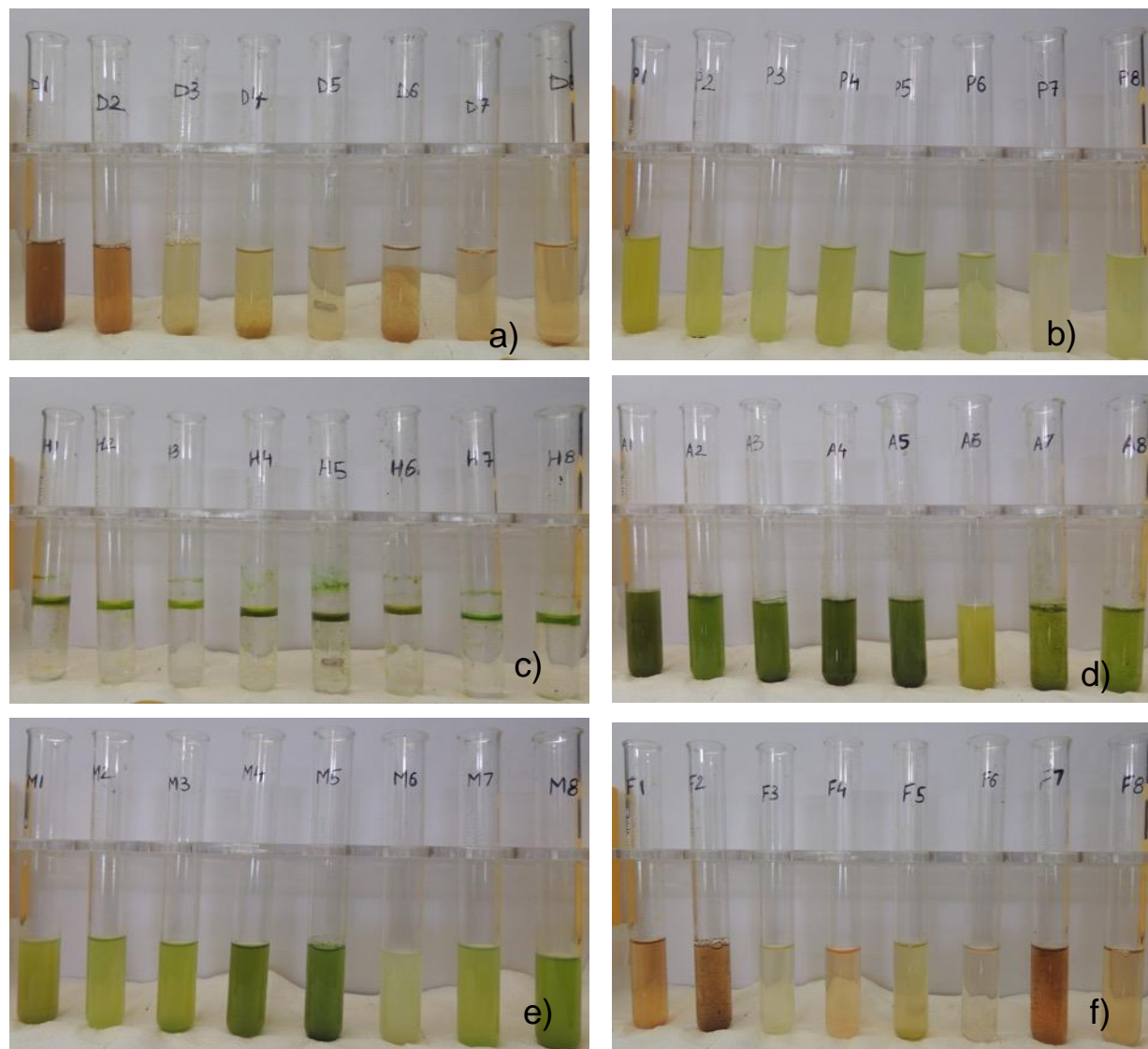




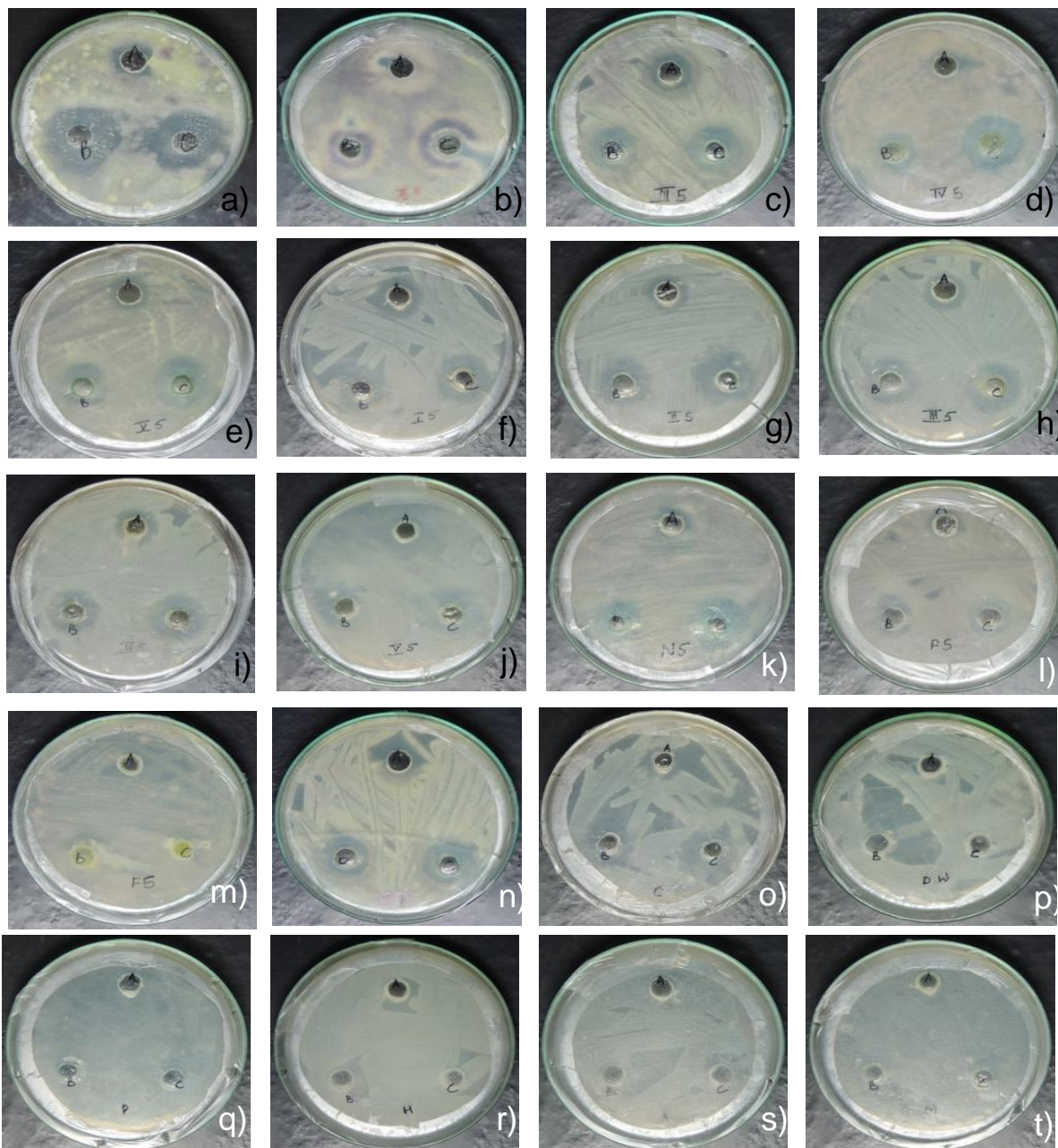
**Fig.4** Green synthesised Copper nanoparticles of *Gliricidia sepium* and *Piper nigrum*, a) dry leaf distilled water extracts, b) dry leaf propane extracts, c) dry leaf hexane extracts, d) dry leaf acetone extracts, e) dry leaf methanol extracts, f) fresh leaf distilled water extracts.



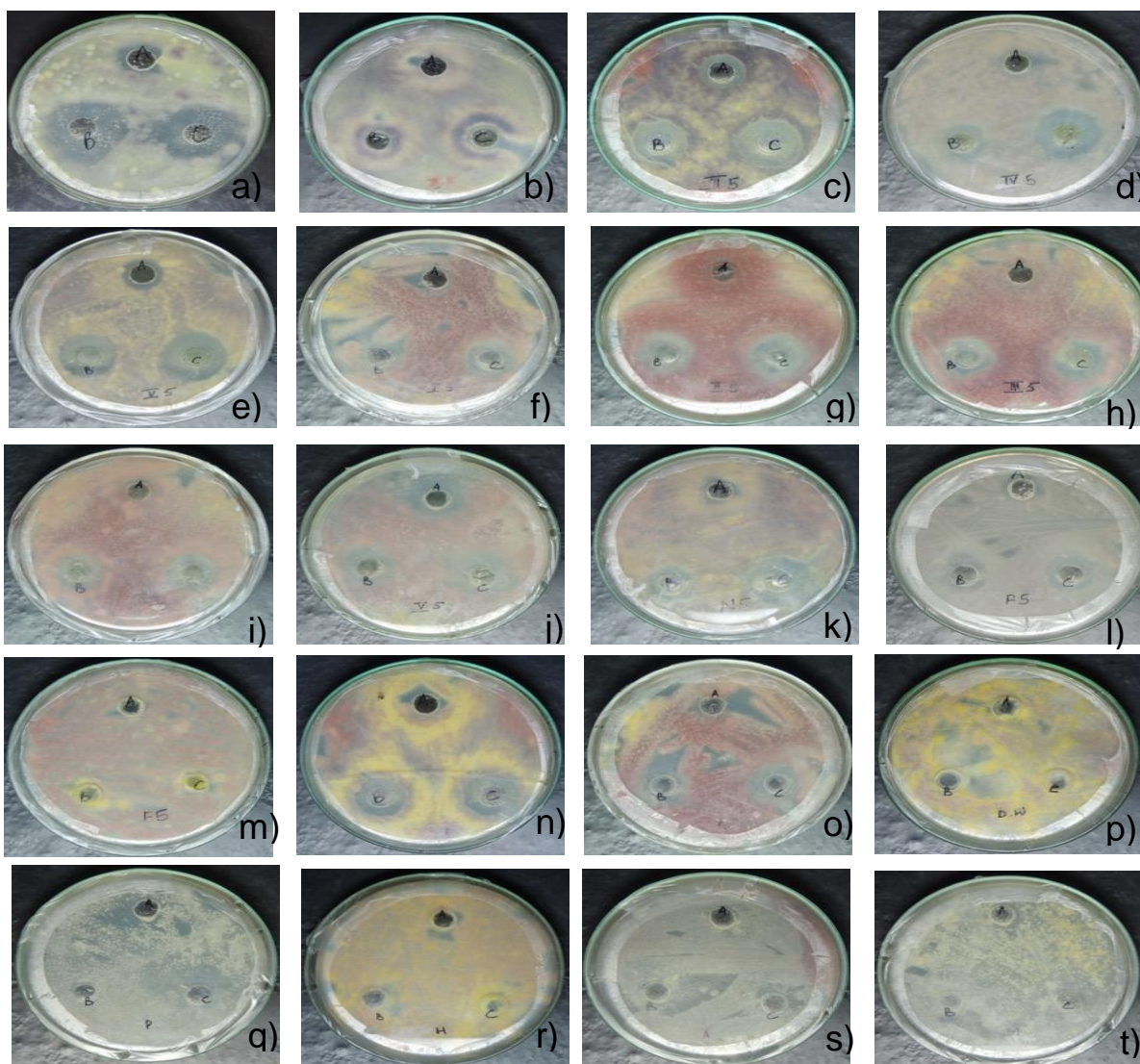
**Fig.5** Green synthesised Zinc nanoparticles of *Gliricidia sepium* and *Piper nigrum* a) dry leaf distilled water extracts, b) dry leaf propane extracts, c) dry leaf hexane extracts, d) dry leaf acetone extracts, e) dry leaf methanol extracts, f) fresh leaf distilled water extracts.



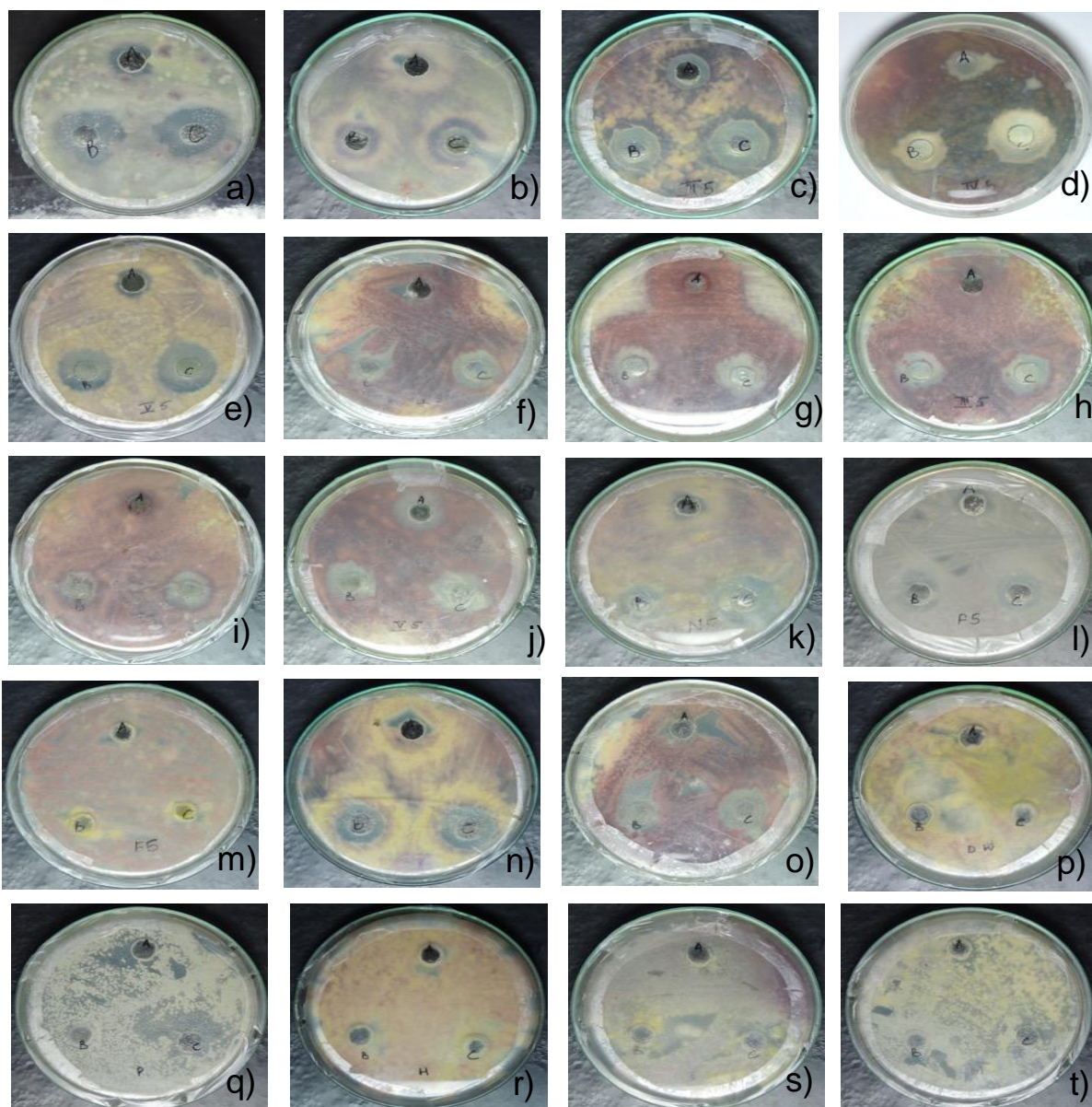
**Fig.6** Antifungal activity study using well diffusion method of Cheemakonna (*Gliricidia sepium*) 24 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150  $\mu$ l), b) Propane, c) Hexane, d) Acetone, e)Methanol,f) to j) above 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).



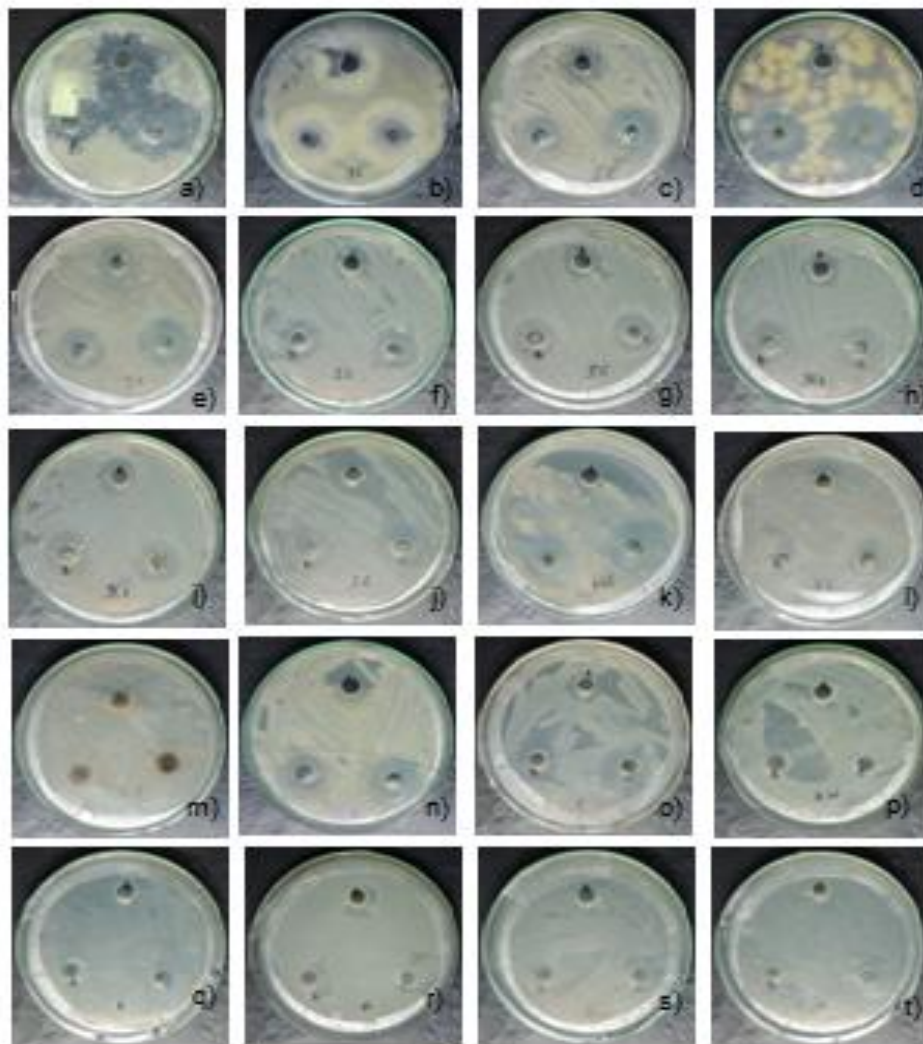
**Fig.7** Antifungal activity study using well diffusion method of Cheemakonna (*Gliricidia sepium*) 48 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150 µl), b) Propane, c) Hexane, d) Acetone, e)Methanol,f) to j) above mentioned 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).



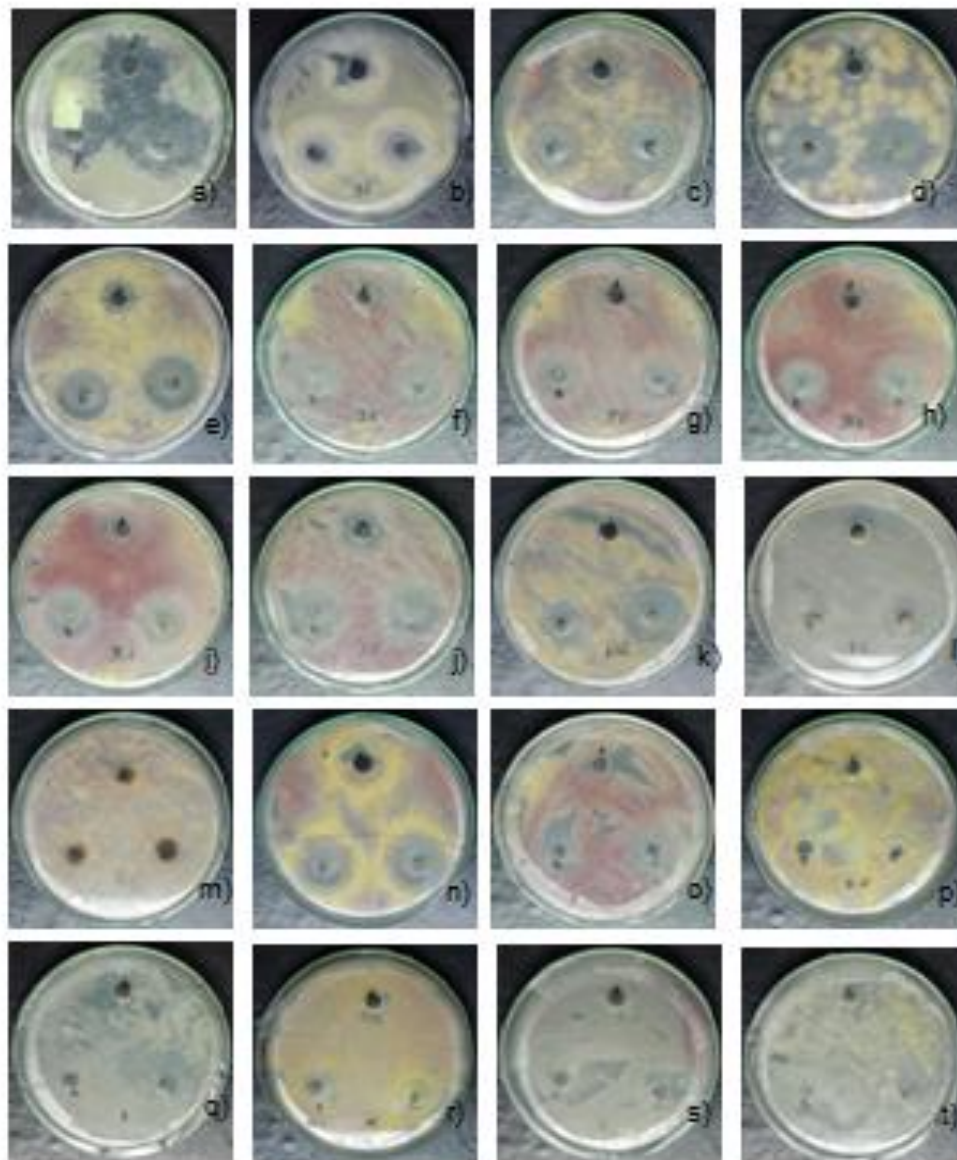
**Fig.8** Antifungal activity study using well diffusion method of Cheemakonna (*Gliricidia sepium*) 72 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150  $\mu$ l), b) Propane, c) Hexane, d) Acetone, e)Methanol,f) to j) above 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).



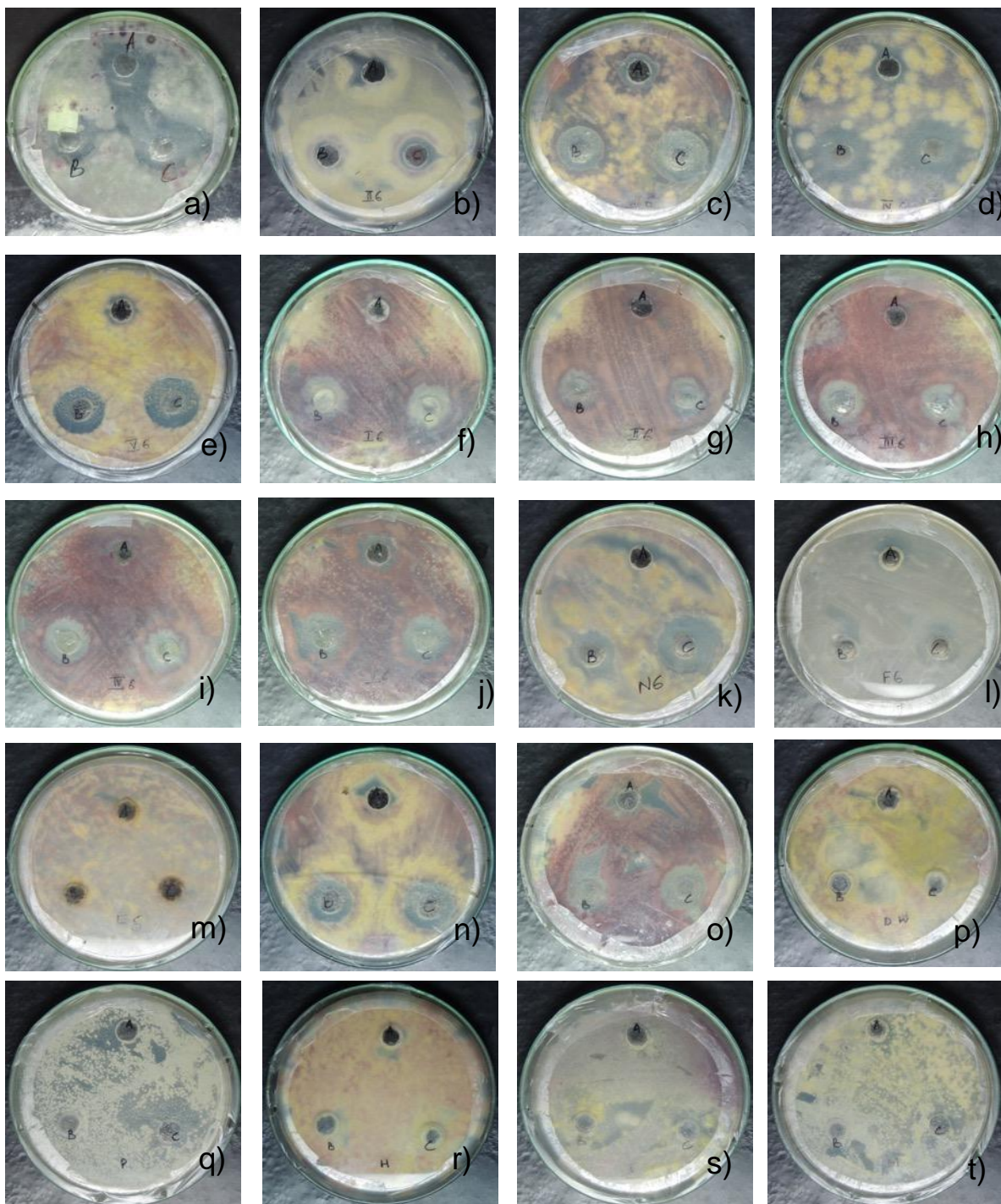
**Fig.9** Antifungal activity study using well diffusion method of Pepper (*Piper nigrum*) 24 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150  $\mu$ l), b) Propane, c) Hexane, d) Acetone, e)Methanol,f) to j) above mentioned 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 Pepper (*Piper nigrum*) 48 hrs leaf extract nanoparticles (Cu) and (Zn) a) green synthesised Cu nanoparticle test plate (Distilled water; 50, 100 and 150  $\mu$ l), 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 Pepper (*Piper nigrum*) 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).





## 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).

## Results and Discussion

### Synthesis of nanoparticles

Nanoparticles were synthesized from the leaf extracts of *Gliricidia sepium* and *Piper nigrum* using solvents like distilled water, propane, hexane, methanol and acetone.

### Copper nanoparticles

Copper nanoparticles were synthesized from leaf extract of different plants (*Gliricidia sepium* and *Piper nigrum*). 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

### Zinc nanoparticles

Zinc nanoparticles were synthesized from leaf extract of different plants (*Gliricidia sepium* and *Piper nigrum*). 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.

### Characterization of nanoparticles

#### 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 *Gliricidia sepium*, *Piper nigrum* 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.

#### 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 *Gliricidia sepium*, *Piper nigrum* with each solvent.

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

### Antifungal assay

The leaf extracts of *Gliricidia sepium*, *Piper nigrum* with solvents distilled water, propane, hexane, methanol and acetone and the fresh extract showed the growth inhibitory effects against *Fusarium oxysporum cubense*.

#### Cheemakonna (*Gliricidia sepium*)

Compared to all the other plants this plant shows the least range of inhibition with a slight change in inhibition zones with copper and zinc nanoparticles. The highest zone formed is 2.6 cm with the zinc nanoparticle with propane extract.

#### Pepper (*Piper nigrum*)

The anti-fungal activity of all the extracts showed a similar range zone of inhibition with both copper and zinc nanoparticles. With water as its solvent, the copper nanoparticles shows steep increase in anti-fungal activity with a zone of inhibition of 3.5 cm with a quantity of 150µl in the agar well. The results shows that leaf extracts of *Gliricidia sepium*, *Piper nigrum* 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 for green synthesis of nanoparticles.

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