"Combinatorial Antimicrobial Activity of *Curcuma longa* and *Glycyrrhiza glabra* Extracts along with Metal Ions on Food Spoilage Bacteria "

Dissertation Report

Submitted in partially fulfillment of the

Requitrement for the award of the

Degree of

Master of Technology in Biotechnology

By

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(2014-2015)

CERTIFICATE

This is to certify that **Gagandeep Kaur** bearing registration no. **11300850** have completed Pre-dissertation project report (BTY 731), entitled **"Combinatorial Antimicrobial activity of *Curcuma longa* and *Glycyrrhiza glabra* extracts along with Metal ions on food spoilage bacteria"** under my guidance and supervision. To the best of my knowledge, the present work is the result of their original investigation and study. No part of the report has ever been submitted for any other degree at any university. The report is fit for submission and the partial fulfilment of the conditions for the award of **M.Tech., Biotechnology.**

Mr. Prabhjot Singh Jassal,

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DECLARATION

I hereby declare that the project work entitled **"Combinatorial Antimicrobial Activity of *Curcuma longa* and *Glycyrrhiza glabra* Extracts along with Metal Ions on Food Spoilage Bacteria"** is an authentic record of my own work carried out at **'Lovely Professional University'** as requirement of dissertation work project for the award of degree of **"M.tech in Biotechnology"** at **Lovely Professional University**, **Phagwara** under the guidance of **"Mr. Prabhjot Singh Jassal, Assistant Professor**, **L.P.U"** during August, 2014 to April, 2015. I hereby declare that no previously published document has resemblance with my work to be identified as under act of Plagiarism. All of the writing and project work in this report is mine. Whenever I have borrowed material from other sources, I have diligently acknowledged the source of the borrowed material.

(Gagandeep kaur)

Registration no. 11300850

Date: 22 April, 2015

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(Gagandeep Kaur)

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List of Abbreviations

Abbreviations	Description
LPU	Lovely Professional University
HHRC	Herbal Health Research Consortium
HPTLC	High Performance Thin Layer Chromatography
USDA	United States Department of Agriculture
16s rRNA	16 Subunit Ribosomal Ribonucleic Acid
MIC	Minimum Inhibitory Concentration
UTIs	Urinary Tract Infections
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
SARS	Severe Acute Respiratory Syndrome
IL	Interleukin
AIDS	Acquired Immuno Deficiency Syndrome
TNF	Tumor Nacrosis Factor
CFU	Colony Forming Unit
HIV	Human Immunodeficiency Virus
Rf	Retention factor
s	Solid
aq	Aqueous
g	Gas
l	Liquid
Cu^{2+}	Copper metal ion
HNO3	Nitric Acid

Units and Measurements

Units	Measurements
°C	Celsius
min	Minute
mm	Millimetre
M	Mole
µl	Micro litre
ml	Millilitre
mg	Milligram
%	Percentage
dl	Decilitre
nl	Nanolitre
sec	Second

Abstract

The present study of antibacterial activity of aqueous and ethanolic extract of *Curcuma longa* and *Glycyrrhiza glabra* was reported against food spoilage bacteria. Aqueous and ethanolic extract of *Curcuma longa* and *Glycyrrhiza glabra* along with metal ions was also conducted to find out the efficacy and potency of the antimicrobial compounds against *Paenibacillus popilliae*. Ethanolic extract of *Curcuma longa* proved maximum activity against food spoilage bacteria. The metal ion showed increased activity along with plant extract at different concentration about 10%, 20%, 30%, 40% and 50%. Aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra along* with metal ion showed maximum antibacterial properties against food spoilage bacteria as compare to the ethanolic extract of plants. Aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* was subjected to get Minimum inhibitory concentration at 0.8mg/ml against food spoilage bacteria. The aim of the study was to evaluate in vitro antimicrobial activity of ethanolic and aqueous plant extract increased by introducing the copper metal ions at different concentration for the prevention of food spoilage bacteria and preservation of food products.

Keywords: Antimicrobial, *Curcuma longa*, *Glycyrrhiza glabra*, Copper metal ion, food spoilage bacteria.

CHAPTER 1

Introduction

Microorganism present in food products may cause food spoilage and poisoning which leads to change texture, flavour and colour of the products and finally generate food borne diseases (Deckar, *et al*., 1996). For the preservation of food products natural antimicrobial agents are widely used for safety purpose and efficacy of food products (Draughon, 2004). Natural products like spices and herbs are very important for human diet. Species and herbs are used for food preservation, medicinal purposes and enhance flavour and texture of food products (Parekh & Chanda, 2007). Antimicrobial agents are present in the plants have antimicrobial activity having chemical structure. Antimicrobial agents widely observed include tannins, terpenes, alkaloids, flavonoids, glycosides and organic acids (Cowan, 1999).

Spices are one of the most common natural products are used for preservation of food products and enhance the flavour texture and improve the shelf life of food products (Nevas *et al.*, 2004; Souza *et al.*, 2005). Medicinal plants have least side effects than antibiotics which are used for the treatment of various infections (Tepe *et al*., 2004). Species are obtained from plant sources used for enhance flavour. Plants sources are stem, bulb, buds, roots, fruits and rhizomes (Mukhtar & Ghori, *et al*., 2012).

Medicinal plant roots are most important sources of secondary metabolities in food and pharmaceutical industry (Shabani *et al*., 2009). Various studies has been reported to prove antimicrobial agents inhibit the growth of microorganism and prove the efficacy of medicinal plants against microbes (Thenmozhi & Rajeshwari, 2010; Sharma & Kumar, 2009).

Antimicrobial agents extracted from the herbs and spices are alkaloids, phenols, steroids, essential oils, glycosides and tannins (Ebana *et al*., 1991). Essential oils and their compounds reported as active agents against the microorganism like food-borne pathogens and spoilage bacteria (Hammer *et al*., 1999; Dorman *et al*., 2000). Spices and herbs used to add into food products not only for preservation food

from spoilage. But also used in medicine preparation purposes and pharmaceutical purposes (Beuchat, 1994; Nakatani, 1994; Cutler, 1995).

Nedorostova *et al*. (2009) was reported that essential oils have antibacterial properties against food borne bacteria- *Escherichia coli* and *Staphylococcus aureus.* Zang *et al.* (2009) was reported that antibacterial properties of essential oils (pepper, nutmeg, liquorice, turmeric, aniseed, cassia bark) against four meat spoilage and pathogenic bacteria.

Curcumin isolated from species *Curcuma longa* (Turmeric) have antibacterial (Chattopadhyay *et al*., 2004; Di Maro *et al*., 2007), antifungal (Chattopadhyay *et al*., 2004); anti-inflammatory (Punithavati *et al*., 2000). The Root of liquorice named as *Glycyrrhiza glabra* is widely used as medicine in worldwide and known as a 'Grandfather of herbs' (Asl & Hosseinzadeh, 2008). Recently research has been reported that licorice has neurological properties like antidepressants, anticovulsant and anxilytic effects (Cho *et al*., 2012).

Copper is the most important element for human system. Copper consists of several factors to perform biological functions. Copper metal ions have ability to reduce the microbial infections which are released by bacteria (Theivasanthi & Alagar, 2011). The high concentration of Copper ions inhibits the growth of microbial activity and produced the toxic effects on microorganism (Faundez *et al*., 2004).

CHAPTER 2

Review of literature

2.1. Medicinal Plants (herbs):

The word "Herbs" derived from the Latin 'herba'. It means medicinal plants. Medicinal plants consist of natural constituents and rich sources of antimicrobial agents. Most of plants used in medicinal purpose in different countries in order to evaluate the powerful drugs. A wide range of medicinal plant parts like stem, roots, fruits and flowers are used having medicinal properties (Newman, *et al*, 2000).

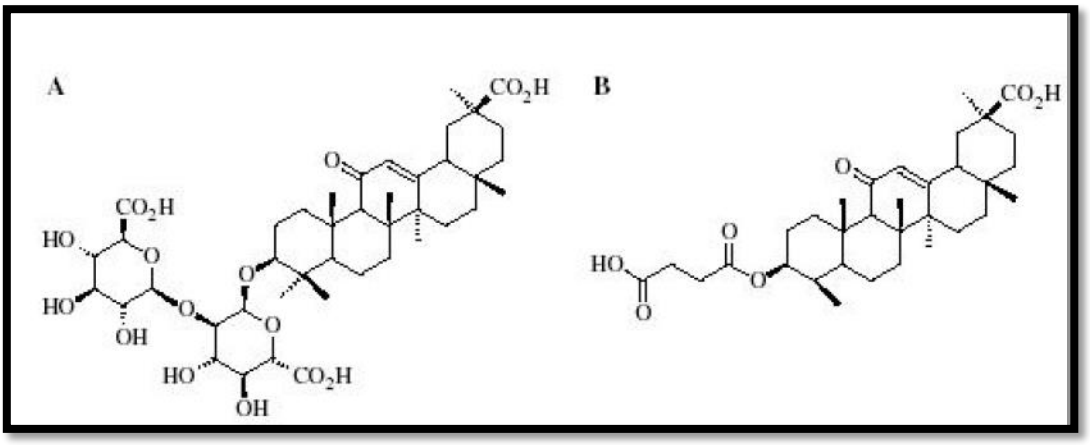
Dhankhar, *et al*., (2013) has been reported that the large amount of phytochemicals are found in spices are isoflavones, anthocyanins and flavonoids are mostly found in spices. Spices have been defined as plant substances having aromatic or with strong taste, used to enhance the taste of foods. The active ingredients of plants used against microorganisms are mostly called as the secondary metabolites (i.e. alkaloids, glycosides etc.) that are widely present in herbs and spices commonly used in Indian food preparations and food industry (Pandey & Singh, 2011).

2.1.1. *GLYCYRRHIZA GLABRA*

Glycyrrhiza word is derived from Greek like glykos, meaning sweet and rhiza, meaning root. In north India, *Glycyrrhiza glabra* is called as Mulaithi and the other name is liquorice. Glycyrrhiza is used in medicinal purposes for cough, colds and painful swellings (Chopra *et al*., 2002).

Figure 1: Roots of *Glycyrrhiza glabra.*

The sweet taste of liquorice root occurs in the presence of the glycyrrhizin (A). Glycyrrhizic acid consists of hydrophillic part having two molecules glucuronic acid, and hydrophobic part having glycyrrhetic acid (Obolentseva *et al*., 1999). Carbenoxolone (B) also has antipyretic (Lata *et al*., 1999), antimicrobial, antiherpes (Ceremelli *et al*., 1996), and anxiolytic (Ambawade *et al*., 2001) activities.



A: Glycyrrhizin B: Carbenoxolone

Figure 2: Structure of Glycyrrhizin and Carbenoxolone; A) Glycyrrhizin and B Carbenoxolone

- **Traditional uses**

Glycyrrhiza glabra roots are widely used for the treatment of anemia, gout, sore throat, tonsillitis, hyperdypsia, fever, cough, skin diseses, sweeling, acidity bleeding and jaundice (Sheth, 2005).

- **Antibacterial & antioxidant activity**

Research reported as the hydro-methanolic extract from the roots of *Glycyrrhiza glabra* showed the presence of phytochemicals like saponins, alkaloids and flavonoids etc because these phytochemical agents showed antibacterial and antioxidant properties. *Glycyrrhiza glabra* is important drugs for prevention of the bacterial infection and scavenging of hydroxyl radicals which are generally produced during carcinogensis (Sharma *et al*., 2013).

- **Anticoagulant**

Glycyrrhizin compound present in the *Glycyrrhiza glabra* having anti-inflammatory activity and found as the first plant based inhibitor of thrombin. The thrombin induced platelets aggregation was observed by the action of glycyrrhizin to be inhibited. Platelet aggregated factor or collagen induced agglutination was not affected by the compound of glycyrrhizin (Mauricio *et al*., 1997; Mendes-Silva *et al*., 2003).

- **Antiviral**

Glycyrrhizin compound showed antiviral activity and inhibit the binding of virus to cell. It has been observed as HIV-1, yellow fever virus and encephalitis virus. Antiviral activity of ribavirin, 6-azauridine, pyraziofurin, mycophenolic acid and glycyrrhizin has been checked against two clinical isolated SARS virus from which patients suffering with SARS (severe acute respiratory syndrome). Glycyrrhizin has been used for the treatment of patients which suffering from HIV-1 and chronic hepatitis C virus (DeClercq, 2000; Badam, 1994; 1997).

- **Mechanism of action**

Glycyrrhiza glabra attributed many beneficial effects and undergoes number of mechanisms like glycyrrhizin and glycyrrhizic acid inhibits the growth and cytopathology of numerous RNA and DNA viruses, including hepatitis A (Crance *et al.,* 1990) & C (Van Rossum *et al*., 1999). *Glycyrrhiza glabra* compounds also exhibit hepatoprotective activity which helps to improve the tissue pathology in hepatitis patients which is done by lowering the serum liver enzyme levels (Van Rossum *et al*., 2001).

2.1.2. *CURCUMA LONGA* (Turmeric)

Curcuma longa, also known as 'turmeric', widely used as colouring agents and species having antimicrobial activity (Luthra *et al*., 2001). *Curcuma longa* is related to Zingiberaceae family (Chattopadhyay *et al*., 2004). Many Researchers investigate antibacterial agents having antibacterial activity found in medicinal plants (Chattopadhyay *et al*., 2004). The compounds are found in turmeric are curcumin (Fig.3) (diferuloyl methane), demethoxycurcumin and bisdemethoxycurcumin

(Chainani, 2003). 95% of curcumin have antioxidant is bioactive agents and posses like anti-platelets, cholesterol lowering antibacterial and antifungal effects.

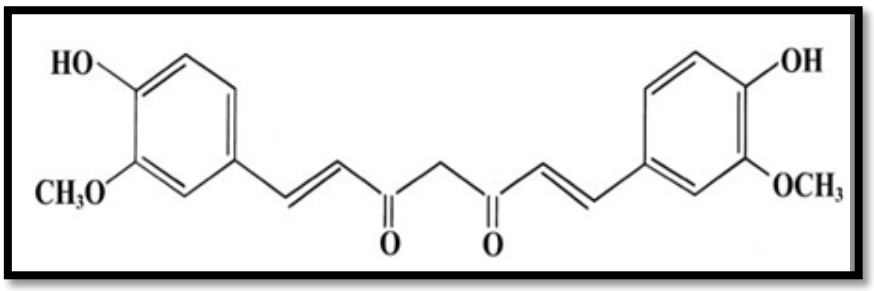


Figure 3: Chemical structure of curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione (Rakshit and Ramalingam, 2010).

Rhizome is part of Turmeric widely used for medicinal purpose. It is cleaned, boiled, dried and grinded. Powder form of turmeric is used as spices (Hakkem, 2006; Cruz & Janero, 2001). Curcumin and oil extract from *Curcuma longa* plant control the growth of several microorganisms like, *Streptococcus, A. parasitius*, A. varies, *Straphylococcus, Lactobacillus* etc (Shankar & Murthy, 1979). Ethanol extract of turmeric showed best result against pathogens as compare to the aqueous turmeric extract (Mukhtar *et al*., 2012).

Turmeric extract showed antimicrobial activity against *B. substilis* and *E. coli* bacteria (Gur *et al*., 2006). Extract from *Curcuma Longa* have antibacterial activity against food poisoning pathogens like *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella typhimurium*, *E. coli* bacteria (Singh *et al*., 2002).

2.1.2.2. Turmeric has chemo-preservative properties: Turmeric plants consist of many compounds containing beneficial results with chemo-preservative properties (Kanai *et al*., 2011).

- **Pancreatic cancer**

Gemcitabine patients suffer from pancreatic cancer diseases (Kanai *et al*., 2011). Gemcitabine with combination of 8gm of curcumin has taken daily which contain curcuminoids with 73% curcumin, 22% of demethoxycurcumin and 4% of disdemethoxycurcumin. Improvement of cancer depend upon the intiation of

curcumin takes 161days. But gemicitabine was in 70 days and result is significant (Epelbaum *et al*., 2010).

- **Diabetes**

Under Placebo controlled 8 weeks study demonstrated that 72 patients suffering from type-II diabetes. Study was carried out, either 300 mg curcumin twice times daily, atorvastatin 10mg daily or placebo received by patients. During post treatment and baseline treatment, endothelial function was evaluated. IL-6, TNF-α, malondialdehyde and endothelin-1 was blood markers analysed and result was obtained significant comparable with both of Atorvastatin and curcumin enhance the endothelial functions (Usharani *et al*., 2008).

- **Digestive disorder**

The study was observed that reduce the effects of ulcerative colitis and chrohn's diseases by curcumin (Holt, Katz & Kirshoff *et al*., 2005). 25 patients suffering from gastric ulcer was held in phase-II trials. 600mg turmeric powder given to patients used five times daily. This result was evaluated that 48% of patients healed had completely after four weeks. After twelve weeks, success rate increased 76% ulcer free during treatment. Neither blood abnormalities nor adverse effects significant had noted (Prucksunand *et al*., 2001).

- **Mechanism of action**

Curcumin isolated from the turmeric protect the DNA against the breaking of single strand which induced by oxygen (Shrinivas *et al*., 1992). Turmeric extract have anti-inflammatory properties. Whenever turmeric administrated orally curcumin inhibit many factors in human system like platelets aggregation, inhibit lymphocyte activity and promote the fibrinolysis and stabilize the lysosomal membrane (Srivastava *et al*., 1995).

2.2. Natural plant extract as Antimicrobial agents

Many researchers have been reported that spices and herbs used as natural antimicrobial agents in food and beverages industry and reduction of contamination has been evaluated. Antimicrobial agents incorporated in packaged system contact

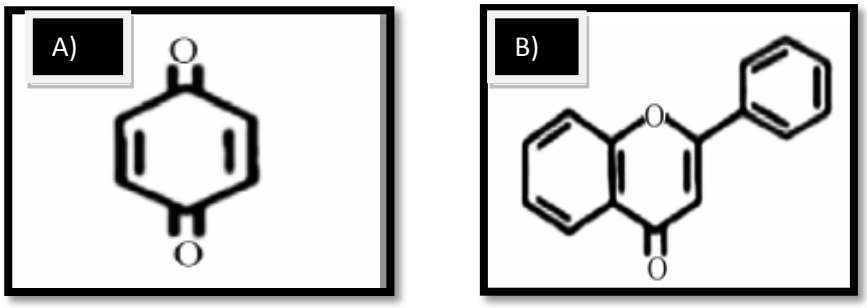
with the surface of food products increasing in demanding and low risk toward the consumer (Nicholson, 1998).

Table 1: Solvents produce active compounds during extraction process **(**Cowan**,** 1999**)**

SOLVENT	ACTIVE COMPOUNDS
1. Water	Tannins, Saponins, Terpinoids
2. Ethanol	Alkaloids, Tannins, Terpenoids, Flavonol
3. Methanol	Terpenoids, Saponins, Tannins, Flavones
4. Chloroform	Terpenoids, Flavonoids
5. Dichloro-Methanol	Terpenoids
6. Ether	Alkaloids, Terpenoids, Coumarins
7. Acetone	Flavonols

Quinones (A) more reactive and coloured compounds and two ketone groups present in aromatic ring. Flavonols, Flavonoids (B) and Flavones are involved in phenolic compounds with one carboxyl groups that inhibit the growth of microorganism. Tannins compounds are polymeric phenolic having astringent activity.

Tannins (C) consists of compounds are soluble in water, alcohol and acetone and cause to precipitate the proteins, crude form of volatile and non-volatile oils extraction from plant's roots, stem, bulb, flower and fruits consists of Flavonoids and isoflavonoids, Saponins, Tannins, Phenolic, Terpenoids (D) and Phenolic acids Pyrones are antimicrobial compounds against pathogen disease (Cowan, 1999; Gurjar *et al*., 2012).



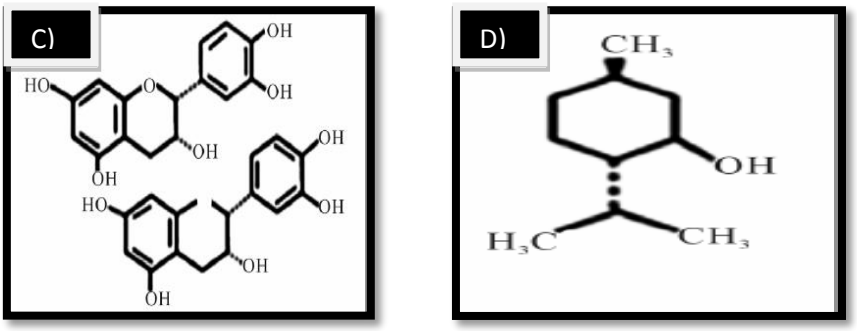


Figure 4: Structure of antimicrobial compounds; A) Structure of quinine, (B) Structure of flavonoids, (C) Structure of tannins and (D) Structure of terpenoids (Gurjar *et al*., 2012).

2.3. Food Spoilage Bacteria:

Food spoilage defined as the undesirable change occurs in food products in the presence of water, air and light promote the growth of microorganism. The different microbes are grown on food products cause food poisoning such as bacteria, fungi and protozoa. After short period of time, Changes in the Food occur cause to lose natural food's ability leads to Perishable food such as meat, fish, milk fruits and vegetables, semi-perishable food like eggs, onion, potatoes, carrot and beans and non perishable food such as cereals, pulses and nuts (Sa'id & Khajuria, 2014).

2.4. Antibiotic resistance bacteria:

Antibiotic are used to treat the bacteria which cause illness and disease. Antibiotic have given to patient for safe health. Many diseases killed the people, with long term use of antibiotic. Antibiotic show no effect on bacteria which are resistant to antibiotic bacteria which survive in the presence of antibiotic called as antibiotic resistance bacteria. Some bacteria resistant to antibiotic naturally such as benzyl penicillin on microorganism present in the human intestine system with low effects (USDA, 1997).

2.5. Mode of Action of Antibiotics:

Ciprofloxacin is broad spectrum antibiotic either for gram positive and gram negative bacteria, belonging to the family of fluroquinolone class. This antibiotic has antibacterial property and stops bacterial infections that linked to enzyme which are

helping in DNA rewinding after being copied which directly leads to stop the synthesis of bacterial DNA and Protein. Ciprofloxacin is broad spectrum antibiotic inhibit the activity of both gram positive and gram negative bacteria. Ciprofloxacin works by inhibiting the activity of DNA gyrase, topoisomerase IV and type II topoisomerase enzymes. Ciprofloxacin separate the strand of bacterial DNA and inhibit the cell division (Drlica *et al*., 1997).

2.6. Metal ions have antimicrobial activity:

Metal ions play essential to modify the flow of electron in a substrate and enzyme they have ability to bind with substrate, along with functional groups in active site and helps in providing the process for redox reactions if metal have number of valence sites (Silva & Williams, 1991).

Metal ions have positive charged particles and having ability to sharing the electrons with other atoms that formed bond and charge-charge interaction. Metal ions act as electrophiles. Metal ions are positively charged have value greater than one. Metal ions have ability react with ligand because of larger volume. Concentration of metal ions is high at neutral pH conditions (Glusker *et al*., 1999).

Ligands are the atom or groups of atom that bind to metal ions due to electrostatic interactions. Ligands may be negative or neutral charged have density to donate electron to metal ions. The coordination of metal ions depends upon the number of ligands atoms bind to it. Mg2+, Ca2+, Mn2+, Zn2+ and Pb2+ are the transistional metal ions (Bock *et al*., 1994).

2.6.1. Copper metal ion

Copper is the most important element for human system. Copper consists of several factors to perform biological functions. Copper have antimicrobial activity has been approved by the US Environmental protection agency. Copper metal ions have ability to reduce the microbial infections which are released by bacteria (Theivasanthi & Alagar, 2011). The high concentration of Copper ions inhibits the growth of microbial activity and produced the toxic effects on microorganism (Faundez *et al*., 2004). The normal average of copper present in human blood is 87 to 153 mg/dl in woman and 89 to 137 mg/dl in men (Lukaski *et al*., 1995).

Antibacterial activity of copper ions depends upon the concentration of ionic copper atoms. Copper ions bind to the amino acid, carboxyl group's leads to denature the proteins. Denaturations of protein by the binding with the copper metal ions cause deactivate the cell surface proteins which are essential in transporting the materials. Copper widely used in food processing industry. Copper and its alloys are used in the purpose to remove food spoilage bacteria (Menkissoglu & Lindow, 1991).

CHAPTER 3

Rationale and Scope of the study

With the evidence of food borne illness and pathogen causing food spoilage associated human disease like salmonella, tetanus, typhoid, cholera, gangrene, bacterial dysentery, diphtheria, tuberculosis, bubonic plague, meningococcal meningitis, pneumococcal pneumonia. These human diseases caused by the contamination and infection of food spoilage bacteria. Hence, it is important to evaluate the natural antimicrobial agents from medicinal plants act as food preservative for food products. The aim to isolate of antimicrobial agents derived from the medicinal plants increase the shelf life of food products for long duration and providing safe health to human beings.

The scope of this study provides many applications in the sector of medical, pharmaceutical industry and widely used in food industry. Spices like *Curcuma longa* and *Glycyrrhiza glabra* can be used to inhibit the growth of antibiotic resistance bacteria and improve the human health condition without any harmful effects. The aim to isolate antimicrobial agents derived from the medicinal plant extract lead to increase the shelf life of food products for long duration and providing safe health to human beings. Natural antimicrobial compounds extracted from medicinal plants are also important for packaging of food products for long time preservation.

CHAPTER 4

OBJECTIVE OF THE STUDY

The aim of our study is to evaluate the antimicrobial activity of medicinal plants and metal ions against food spoilage bacteria which directly leads to cause poisoning and spoilage of many food products. It is important to introduce the natural antimicrobial agents in order to increase the shelf life of food products, providing safe health to human beings.

The objectives of this study to investigate:

1. To isolate bacteria from spoilage food product.
2. To evaluate the antibacterial activity of aqueous and ethanolic extract of *Glycyrrhiza glabra* and *Curcuma longa* on food spoilage bacteria.
3. To evaluate the activity of metal ion on food spoilage bacteria.
4. To evaluate combinatorial effects of metal ions and plant extract.

CHAPTER 5

Material and Research Methodology

Materials and Equipments:

Table 2: **Lists of Instruments and Equipments used in laboratory.**

S. NO.	EQUIPMENTS	COMPANY
1.	Weighing machine	VIBRA J-150S
2.	Incubator	LABFIT
3.	Oven	NSW-143
4.	Autoclave	NSW-227
5.	Laminar air flow Cabinet	NSW-1S09001
6.	Microscope	MAGINUS-10L632
7.	Magnetic Stirrer Machine	LABFIT
8.	Colony Counter Machine	LABFIT
9.	Micropipette	1SO:9001
10.	Microwave oven	IFB-30SC3
11.	Refrigerator	LG
12.	Micropipette	P' Fact A
13.	Micro tips	TARSON
14.	Weighing Balance	Adventure

Table 3: List of Materials, Reagent and Chemical used in the Laboratory.

S. NO.	CHEMICAL NAME	COMPANY NAME
1.	Nutrient Broth	HI-MEDIA
2.	Nutrient Agar	HI-MEDIA
3.	EMB Broth	HI-MEDIA
4.	EMB Agar	HI-MEDIA
5.	Nitrate Broth	TITAN-BIOTECH LTD
6.	Urea Broth	TITAN-BIOTECH LTD
7.	Simmon Citrate Agar	HI-MEDIA

8.	Tryptone Broth	TITAN-BIOTECH LTD
9.	Peptone Water	LOBA-CHEMIC
10.	MR-voges prauskeaur	HI-MEDIA
11.	Copper metal powder	CAS
12.	Nitric acid	CAS
13.	Methanol	LOBA-CHEMIC
14.	Ethanol	CHANGSHU-YANGYUAN CHEMICAL

5.1 Medicinal Plant Collection

Rhizome of Turmeric (*Curcuma longa*) was collected from Health herbal research consortium (HHRC) in Amritsar city and liquorice (*Glycyrrhiza glabra*) was collected from lovely professional university (LPU) in Jalandhar in Punjab state of India.

5.1.1. *Curcuma longa* (Turmeric)

The rhizome of *Curcuma longa* was boiled and dried for 3 days in the presence of sunlight. The dried form of *Curcuma longa* rhizome (Fig 5) was grinded in the electric grinder machine. The powder form of and *Curcuma longa* was obtained (Jangale *et al*., 2012).

Figure 5: Dry rhizome of *Curcuma longa*

5.1.2. *Glycyrrhiza glabra*: Roots of *Glycyrrhiza glabra* was and grinded in the electric grinded machine. Powder form of *Glycyrrhiza glabra* was obtained.

Figure 6: Powder form of *Curcuma longa* and *Glycyrrhiza glabra*.

5.2. Extraction Procedure: *Curcuma longa* and *Glycyrrhiza glabra* extract was prepared by the Soxhlet method.

5.2.1. Soxhlet method:

Curcuma longa and *Glycyrrhiza glabra* was obtained by using solvents in Soxhlet method. The powder form of *Curcuma longa* placed in thimble. Weight of *Curcuma longa* powder was placed in an extraction chamber and placed above the flask containing solvent (ethanol) just below the condenser. Electric heater started to heat the flask and evaporated the solvent at specific temperature conditions and moved toward the condenser.

Condenser helps to form liquid that jumped into extraction chamber consists of plant materials. Design of Soxhlet provide the solvent present in the extraction chamber fallen down in boiling flask. Duration of extraction depend upon the plant material which was used in the soxhlet method. Final step was to evaporate the solvent from plant extract with the help of rotary evaporator. It is easy and quick method to evaporate the solvent from plant extract without any degradation of the extract' metabolities. The weight of final plant extract was calculated and its percentage yield (Naz *et al*., 2010).

5.2.2. Filtration method:

Aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* were prepared by Filtration method. Dry powdered spices of *Curcuma longa* and *Glycyrrhiza glabra* were dissolved into 250ml of distilled water (Fig. 3) and kept at room temperature for 24 hours, then were filtered with help of filter paper. Filtrate was obtained and heated at 40-50°C by using water bath. Then thick paste of *Glycyrrhiza glabra* was obtained.

This thick paste indicates 100% of concentrated extract. These all extracts were stored in refrigerator at 4°C and diluted in different concentration by mixing the different concentration in distilled water (Chhillar *et al*., 2013; Mukhtar and Ghori, 2012) (Fig. 7). Percentage yield of dried extract of plant material was estimated by the following formula (Kumari et al., 2014).

$$\text{Percent Extractive} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant material}} \times 100$$

(Kumari *et al*., 2014).

Figure 7: Aqueous extract solution of *Glycyrrhiza glabra*

5.3. Isolation of bacteria from Spoilage Food Product

Microorganism grows on the various food products like bakery products, milk products (cheese, butter), food and bread. Some factors are responsible to grow in the presence of chemical, physiological and environment factors. Water is the major source for the growth of microorganism. The presence of microorganism in food products creates food borne disease (Jones *et al*., 2006).

- Take beaker (500ml) cereals based products, Pasta used to spoilage in the presence of moisture and covered by brown paper.
- It takes 2-3days to spoilage Pasta product. Microorganisms were grown on the Pasta containing cereal Products (Jones *et al*., 2006).

Table 4: Environmental Factors responsible for spoilaged food products.

S. No.	No. of days	Temperature	Humidity
1.	First day	35°C	55%
2.	Second day	37°C	61%
3.	Third day	36°C	55%

5.4. Preparation of Inoculums and Media preparation:

Microorganism was isolated from cereal food product pasta by pick up the two or three colony with the help of inoculation loop and transfer in nutrient broth. Culture kept in incubator at 37°C for 24 hours (Gour *et al*., 2014). After this, loop transfer in screw capped tubes and streaking was done onto the nutrient agar, incubated at 37°C for 24 hours (Gour *et al*., 2014)

5.4.1. Media Preparation:

- Nutrient Broth: 0.13gm of nutrient broth was measured by measuring machine and dissolved in the 10ml of distilled Water and added into one screw capped tube. Nutrient broth was sterilized by the autoclave at for 15min.
- Nutrient Agar: 1.68gm of nutrient agar was dissolve in 60ml of distilled water. Prepared media was allowed to sterilize by autoclave method for 15 min at 121°C (Singh & Jain, 2011).

5.5. Morphological Tests:

Gram staining used to differentiate the bacteria organism into two groups that is gram positive and gram negative bacteria. Basic steps should be followed to different the gram staining bacteria.

- Fresh bacterial cell was taken from nutrient agar plates with help of inoculation loop and fixed on microscopic slide with slightly low heat of lamp.
- Crystal violet reagents, iodine solution, 95% ethanol and safranin were used in gram staining.

- Crystal violet dye flooded on the slide for 1 min and the slide was wash gently with tap water.
- Secondly, gram iodine solution was flooded on slide for 1 min and washed under tap water.
- 95% ethanol used for decolourization for 30 seconds and washed with tap water.
- Finally drops of safranine was placed on the smear part of bacteria and kept for 1 min and washed under running water.
- Slide was viewed under microscope under oil-immersion. Gram positive bacteria were appeared in blue or violet and gram-negative bacteria were appeared in pinkish red (Murray *et al*., 1999; Baron *et al*., 1994)

5.6. Biochemical Tests: Biochemical test used to identify the bacteria species.

➢ **Methyl Red test and Voges- proskauer test.**

- Take media of MR-VP broth about 10 ml added into the test tubes and 5ml added into one tube and 5ml added into second tube.
- Take inoculums with the help of loop and mix into broth in one tube and second tube was uninoculated. One Inoculated tube act as positive control and other uninoculated tube act as negative control. Kept both tubes into incubator for 24 hours at 30-37°C (Fall, 2011).
- After incubation: for methyl red test added 6-8 drops of methyl red reagents. For voges-proskauer test: 12 drops of Barritt's A and 4 drops of Barritt's B were added, mix for 1 min (Fig 15, 16). (Fall, 2011).

➢ **Urease Broth test:**

- Urease broth was taken in two test tubes. One tube acts as positive control and other act as negative control.
- Pure culture had to be added into positive control and incubate for 35°C for 24 hours Results were observed after incubation (Fig 17) (MacFaddin, 2000).

➢ **Tryptone broth test**:

- Indole test was carried out by using tryptophan broth media. Tryphtophan broth was taken in two test tubes. One tube kept for positive control and other for negative control.

- For positive control, tryptophan broth was inoculated with culture and incubated for 24 to 48 hours at 20°C-24°C. After incubation, 5 drops of Kovac's reagents was added into positive control. If positive result was observed test completed (Fig 18)(Krieg and Holt, 1984; Lennette *et al*., 1980).

➢ **Indole test:**

- The peptone water was inoculated with test organism culture and incubated at 37°C for 24-28 hours.
- kovac' reagents about 0.5 ml added into the test tube. Positive and negative result was evaluated.
- Positive result was observed by the formation of red ring in upper phase and negative result was observed yellow colour (Fig 19) (MacFaddin, 2000).

➢ **Eosin Methyene Blue (EMB) Media:**

EMB Agar used as selective media for gram negative bacteria like *E. coli*, *Paenibacillus popilliae* species.

- Preparation of nutrient broth was streaked on the EMB Agar plates and incubated for 24-48 hours at 35-37°C. Colonies was observed on the area of streaking (Fig 20) (Howard, 1994).

➢ **Simmon Citrate test:** Some organism used citrate for growth and metabolism. Growth of organism was observed on the simmon citrate agar. Citrate used by the bacteria in alkaline conditions. Ph indicator present in the simmon citrate agar that is bromothymol blue was turn to change the colour of media into blue (from acidic into alkaline conditions).

- Petriplates contained simmon citrate agar inoculated by culture and incubated For 18-24 hour.
- Positive result indicates colour of agar was changed from green into blue and negative result was observed no colour change in agar media (Fig 21) (Krieg and Holt, 1984; Lennette et al., 1980).

5.7. Preparation of Copper metal ion stock solution:

Copper metal is reddish brown in colour widely used for the purpose of electrical wires and in plumbing. Copper metal is oxidized by adding nitric acid (HNO_3) and generate Cu^{2+} metal ions.

3Cu(s) + 8HNO3 (aq) ——> 3Cu (NO3)2(aq) + 2NO (g) + 4H2O (l) (Wilkinson & Cotton, 1988).

Figure 8: Preparation of copper metal ion solution.

Cu^{2+} metal ion product initially coordinated to nitrate ions formed from the nitric acid. Firstly giving green in colour and then change into green-brownish colour. This solution diluted into water, water molecules displaced nitrate ions around copper ions in the coordination sites and finally solution turn blue in colour (Fig. 9). 0.5 g copper metal powder dissolved in 50ml of dilute nitric acid (3 M, HNO_3). Solution of copper metal ions was prepared (Wilkinson & Cotton, 1988).

- 10µl stock solution of Cu^{2+} metal ion dissolved in 1ml of water that is concentration 10µl/1ml was used.
- 0.87µl/ml to 1.53µl/ml is used according to the normal concentration of copper in human blood.

➢ **For combinatorial antibacterial activity of medicinal plant extract and copper metal ion stock solution was prepared by following procedure.**

- 1% of copper metal ion was used to check the combinatorial antibacterial activity with medicinal plant extract. Nutrient agar was used and poured into Petri plate. After solidification of agar, 50µl of inoculums culture spread on agar plate with the help of spreader and kept for 5min.
- 5mm diameter of Wells were bored with the help of borer and made 50µl with different concentration by extract with copper metal ion. First well was filled with 10% (45µl extract + 5µl metal ion) and made 20%(40µl extract + 10µl metal ion), 30% (35µl extract + 15µl metal ion), 40% (30µl extract +

20µl metal ion extract) and 50%(25µl extract + 25µl metal ion) and plates was incubated at 37°C for 24 hours (Pandey & Singh, 2011).

5.8. Qualitative Phytochemical Screening of *Curcuma longa* and *Glycyrrhiza glabra*'s extract: Phytochemical Screening was carried out by various standard procedures to identify the presence of chemical constituents in Plant's extract.

- **Saponin test:**

- Take 3 ml of extract was added to test tube and 12ml of distilled water added into it.
- Test tube agitated well by vortex machine. Formation of foam indicates the presences of saponin (Sawant & Godghate, 2013).

- **Flavonoid Test:** Alkaline reagent test

- Take 2 ml solution of extract added into the test tube and 10% of NaOH solution added into it.
- This test showed yellow colour indicates the presence of flavonoid in extract (Sawant & Godghate, 2013).

- **Terpenoids Test:**

- Take 2ml solution of extract added into test tube. 1 ml of chloroform added into it. After this, carefully 1 ml of concentrated sulphuric acid added into test tube.
- This test showed reddish brown colour at interface indicates positive result of terpenoid (Uthayarasa *et al*., 2010).

- **Alkaloid Test**: 3 ml of extract solution was added into test tube and 1 ml of HCL was heated and cooled. After this, filter is done and filtrate was used for alkaloid test.

- Dragendroff's test: 2 drops of dragendroff's was added to extract solution showed creamy precipitate indicates the presence of alkaloids (Sawant & Godghate, 2013).

- **Coumarin Test:**

- Take 1ml of extract solution was added into test tube and make 1ml of 10% of NaOH was added into test tube.
- This test showed yellow colour indicates the presence of Coumarin constituents (Firdouse & Alam, 2011).

- **Anthocyanine Test:**

- Take 2ml of extract solution added into test tube. After this, few drops of sulphuric acid was added into test tube.
- This test showed yellowish-orange colour indicates the presence of Anthocyanin compound (Firdouse & Alam, 2011).

- **Tannin (Lead acetate Test):**

- Take 2ml of extract solution was added into test tube and make 10% of lead acetate.
- Few drops of 10% lead acetate was added into test tube showed white precipitate indicate the presence of Tannin compound (Prabhakar *et al*., 2013).

- **Steroid (Salkowshi Test):**

  - Take 2ml of extract solution into the test tube and 2ml of chloroform was added into it.
  - After this, 1ml of sulphuric acid was added and shaken well showed reddish brown in colour at interface indicate the presence of steroids. Secondly appearance of yellow colour indicates the presence of triterpenoids (Deb *et al*., 2013).

5.9. Screening of Antibacterial Activity by Agar Well Diffusion Method:

- Antibacterial activity of all Aqueous and methanol of *Curcuma longa* and *Glycyrrhiza glabra* extract of liquorice extracts were tested by agar well diffusion method (punithavathi, *et al.*, 2000).
  - The culture plates were prepared by using Nutrient agar of 25ml pouring into each sterile petriplates.
  - The tested bacteria were swabbed over the agar media by using sterile cotton swabs. This provided uniform distribution of bacteria over agar media. About 5mm diameter of wells was made into the agar media with the help of sterile cork borer.
  - Three wells were made into the agar media that is one for extract, second for antibiotic used as positive control and third was filled with negative control used as ethanol and water. The antibacterial activity of assay plates was incubated at 37°C for 24 hours.

- The zone of inhibition were observed around each wells measured as antibacterial activity.

➢ Antibacterial activity of Metal ion such as copper was evaluated against Food spoilage bacteria by Well diffusion method.

- The cultured plates were prepared by using Nutrient agar of 25ml in each petriplates.
- Swabbing was done by using sterilized cotton dip into culture broth and stay for 5min to proceed. Three wells were made with the help of borer.
- One well was filled with negative control distilled water. Other well was filled with high concentration and other was filled with low concentration (Junior & Zanil, 2000).

5.10. Minimum inhibitory Concentration (MIC): Minimum inhibitory concentration is defined as the minimum concentration of antimicrobial agents will used to inhibit the visible growth of microorganism after time of incubation (Pandey & Singh).

- MIC is done by the agar diffusion method. Ethanol and Aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* was used to incorporate into well with different concentration from 1mg/ml to 0.7mg/ml for the determination of minimum inhibitory concentration.
- 10µl of bacterial culture was spread on the nutrient agar plate and kept for 5min. After this, 1mg/ml to 0.7mg/ml minimum concentration was taken to inhibit the growth of isolated bacteria.
- Plates were incubated at 37°C for 24 hours. The minimum concentration of extract and metal ion was evaluated as agar plate with minimum concentration without growth of microorganism (Akerele *et al*., 2007).

5.11. Molecular characterization of bacteria:

The results of molecular characterization of isolate bacteria were obtained by 16s rRNA sequencing from yaazh xenomics center, new Mumbai in Maharashtra. DNA sequencing is the identification of nucleotides in the region of DNA molecule. 16s rRNA gene generally is sequence because it contain variable and conserved regions

among different bacterial species. Databases of 16s rRNA are analyzed and isolated bacteria are determined by comparisons with sequences.

5.12. Estimation of Curcumin content in *Curcuma longa* rhizome by HPTLC:

Estimation of Curcumin content in *Curcuma longa* was obtained from Herbal Health Research Consortium, Amritsar in Punjab. Ethanolic extract of Curcuma longa was selected to check the curcumin content because of maximum antibacterial activity against food spoilage bacteria. The HPTLC method for the simultaneously analysis of curcumin, demethoxycurcumin and bis-demethoxycurcumin from *Curcuma longa* reported here is very simple and suitable for the screening of large number compounds. Rf values for identification of curcumin, demethoxycurcumin and bis-demethoxycurcumin was evaluated by Paramasivam *et al*, 2008. Four parameters are most important to be followed during HPTLC analysis of curcumin, demethoxycurcumin and bis-demethoxycurcumin compounds i.e. spray gas: inert gas, sample solvent type: ethanol, dosages speed: 150 nl/s and Predosage volume: 0.2µl.

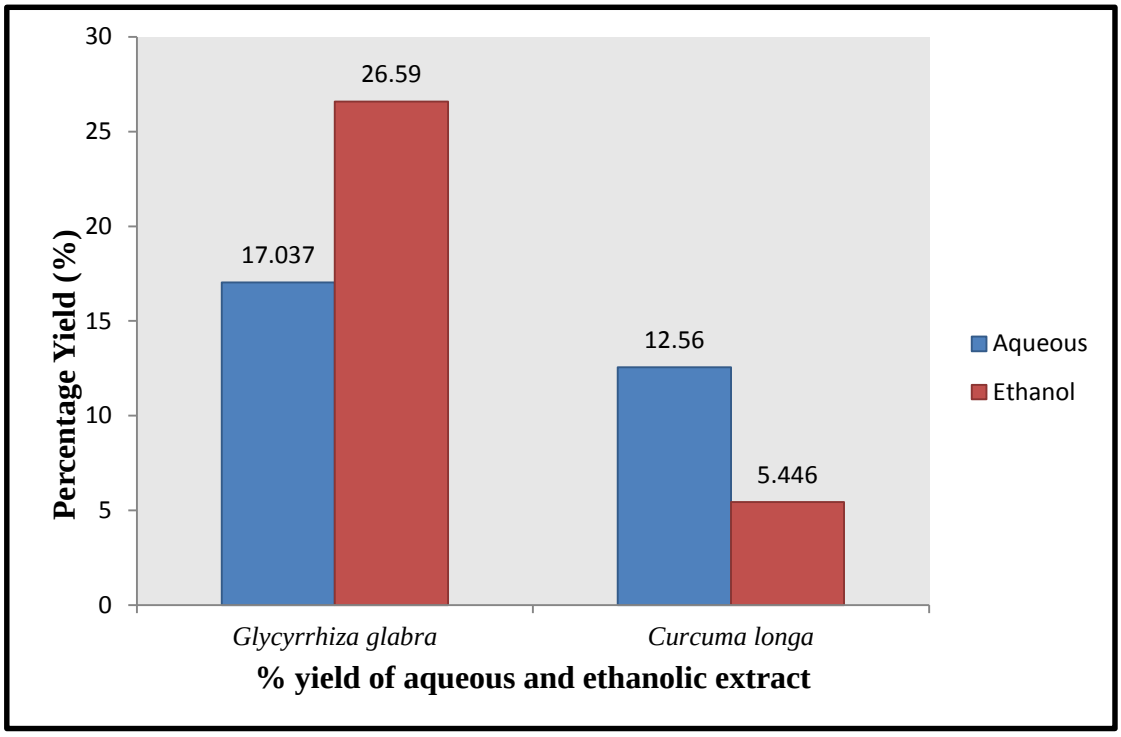
CHAPTER 6

Results and Discussion

6.1. Percentage Yield of *Curcuma longa* and *Glycyrrhiza glabra*: Percentage yield of aqueous extract of *Glycyrrhiza glabra* and *Curcuma longa* was calculated as 17.037% and 12.56%. Percentage yield of ethanolic extract of *Glycyrrhiza glabra* and *Curcuma longa* extract was obtained as 26.59% and 5.446%.

Table 6: Percentage yield (%) of aqueous and ethanolic extract of *Glycyrrhiza glabra* and *Curcuma longa*

Medicinal Plant	Weight of powder (gm)	Solvent	Weight of extract (gm)	Percentage yield (%)
Glycyrrhiza glabra	106	Water	29.136	17.037%
Glycyrrhiza glabra	70.511	Ethanol	18.748	26.59%
Curcuma longa	60	Water	7.536	12.56%
Curcuma longa	116.718	Ethanol	6.357	5.446%



Graph 1: Percentage yield of aqueous and ethanolic extract of *Curcuma longa* and *Glycyrrhiza glabra*.

Graph 1: y- axis and x-axis of graph showed the percentage yield and aqueous, ethanolic extract of *Curcuma longa* and *Glycyrrhiza glabra*.

6.2. Isolation of Pathogenic Food Bacteria: Food spoilage bacteria isolated from spoilaged pasta food product was shown in Fig 9.

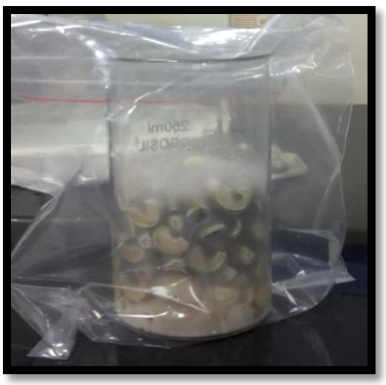


Figure 9: spoilaged of pasta food product.

Colony was taken from spoilage pasta and transfer to Nutrient Broth. Number of colony of bacteria was calculated by serial dilution method. Colony of bacteria was calculated by using colony count machine (Fig.10,11,12,13) (Wiegand *et al*., 2008).

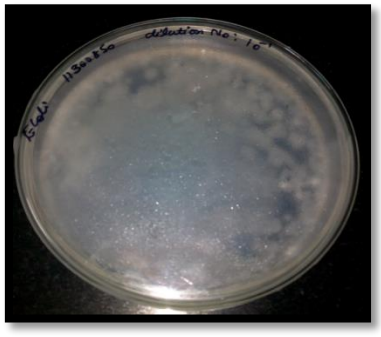


Figure 10: Dilution no.1

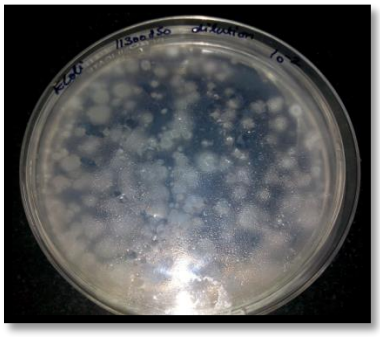


Figure 11: Dilution no. 2

Figure 12: dilution no. 3

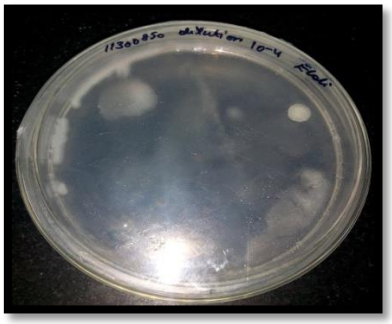


Figure 13: dilution no. 4

Dilution no. 1 (Fig. 12) was containing 166 colony, Dilution no. 2 (Fig. 13) was containing 159 colony, dilution no. 3(Fig. 14) was evaluated 92 colony and dilution no. 4 (Fig. 15) was contained 77 colony.

N= No. Of colony per plate * 10/ 10^{-D} (CFU/ml)= 16600 cells/ml in dilution 1.

6.3. Morphological Test:

Gram staining test was done for morphological characterization. under microscope at 100X with oil immersion. Fig 14 showed blue-violet rod shaped gram negative bacteria when viewed under 100X oil immersion.

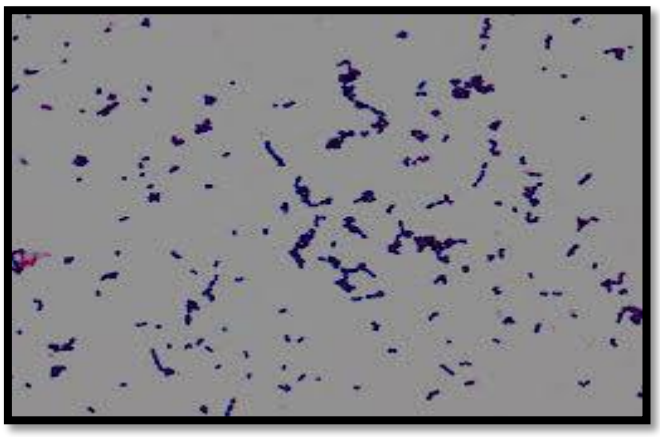


Figure 14: Microscopic view of gram positive bacteria.

6.4. Biochemical Tests: The results of biochemical tests showed in following figures.

Figure 15: Methyl red test. Figure 16: Voges-Proskauer Test

Figure 17: Urease Positive and Negative test. **Figure 18**: Tryptone broth negative and positive test

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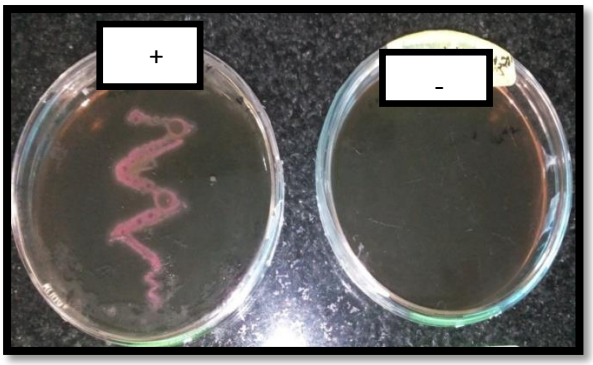


Figure 19: Indole positive and negative test. **Figure 20**: Isolated bacteria on EMB agar.

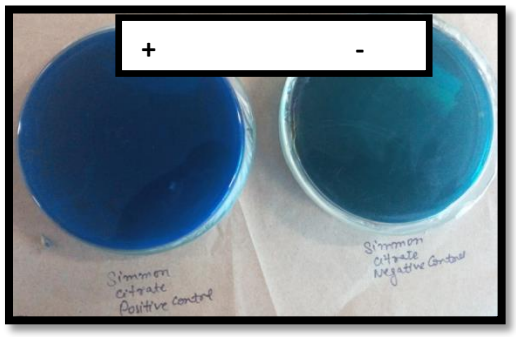


Figure 21: Simmon citrate test in test tube. **Figure 22**: Positive and Negative results of Simmon Citrate Agar petriplates.

Table 7: Lists of Biochemical tests with positive and negative results**.**

S.No.	Biochemical Tests	Positive Result	Negative Result
1.	Methyl Red	-	-
2.	Voger-prouskauer	-	-
3.	Urease	-	-
4.	Tryptone broth	-	-
5.	Indole test	-	-
6.	EMB agar	+	-
7.	Simmon citrate	+	-

(+) = positive test; **(-)** = negative test.

Table 7 showed that selective media EMB agar and Simmon citrate utilization test was given as positive result for the presence of *Paenibacillus popilliae*.

6.5. Phytochemical Screening of *Curcuma longa* and *Glycyrrhiza glabra* extract: various standard tests were performed to identify the presence of chemical constituents act as antimicrobial agents. Different antimicrobial constituents like Saponins, tannins, flavonoids, alkaloids, Anthocyanin, coumarin, terpenoids and steroids were evaluated by performing various tests.

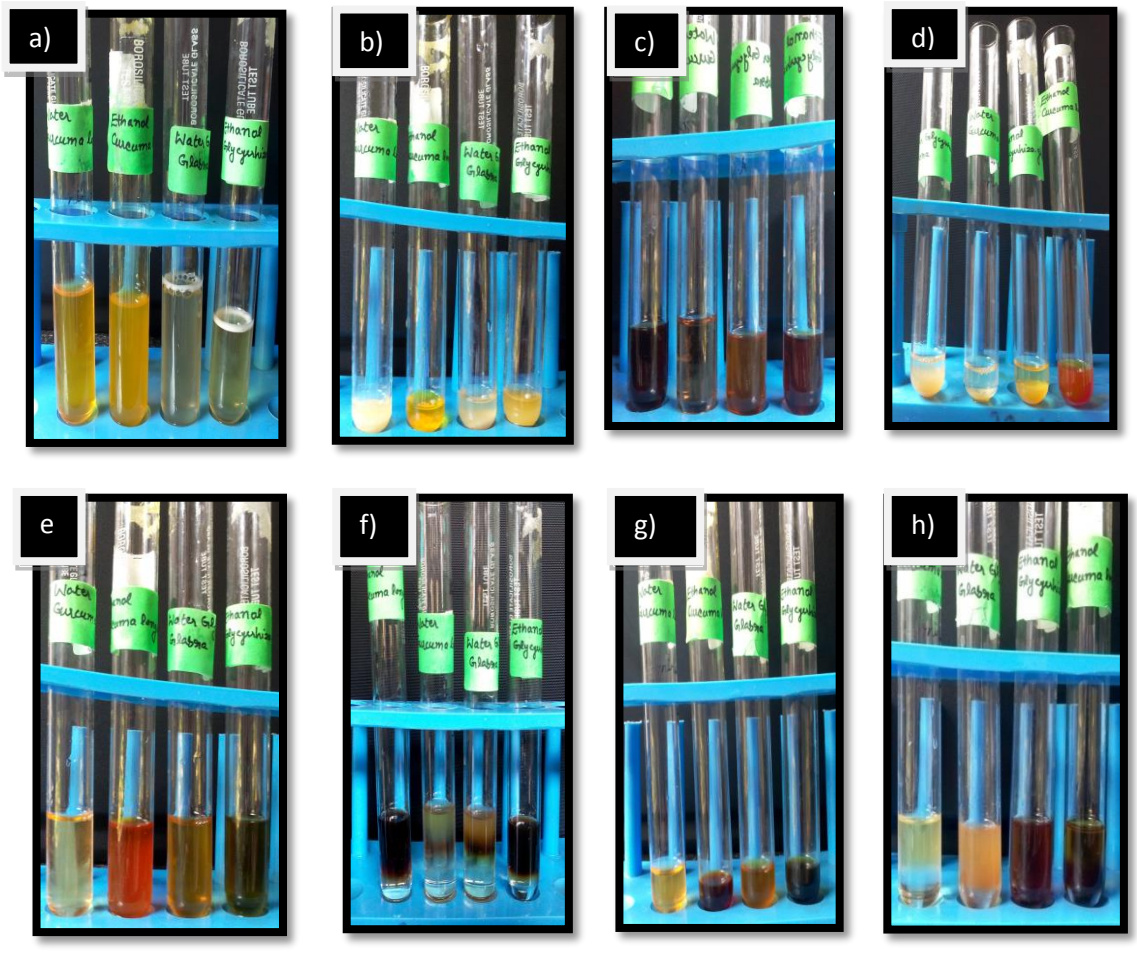


Figure 23: Results of phytochemical tests: a) Saponin Test; b) Flavonoid Test; c) Tannin Test; d) Alkaloids Test; e) Anthocynine Test; f) Steroid Test; g) Coumarin and h) Terpenoid.

Table 8: Qualitative Phytochemical Screening of Medicinal Plant extract:

Phytochemical Test	*Glycyrrhiza glabra*		*Curcuma longa*	
	Aqueous extract	**Ethanolic extract**	**Aqueous extract**	**Ethanolic extract**
A) Saponin (Frothing Test)	+	+	-	-
B) Flavonoid (Alkaline reagent Test)	-	+	-	+
C) Tannin	-	-	-	-
D) Alkaloids (Dragendroff's Test)	+	-	+	-
E) Anthocyanin	+	-	-	++
F) Steroid	+	+	+	+
G) Coumarin	-	-	+	-
H) Terpenoid (Salkowshi Test)	-	-	-	-

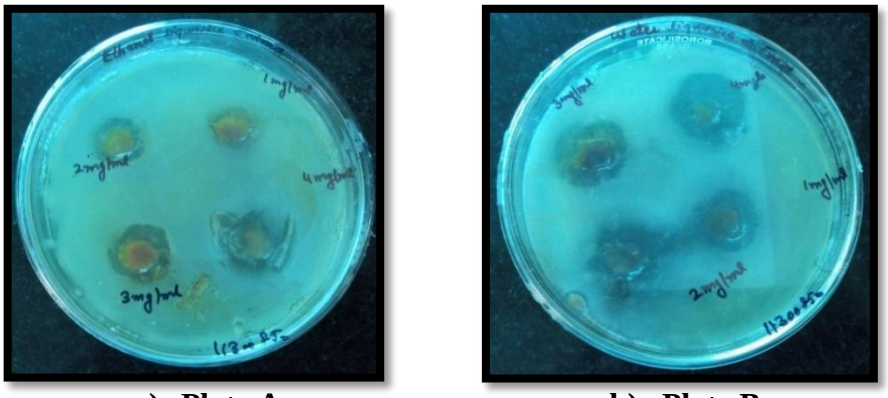
(+) = positive test; (-) = negative test; (++) = moderately present.

Table 8 showed positive negative results of phytochemical compounds. More positive result of steroid was obtained in aqueous and ethanolic extract of plants. Double positive result of anthocyanin was appeared in ethanolic extract of *Curcuma longa*. Tannin compounds showed negative result in aqueous and ethanolic extract of *Curcuma longa* and *Glycyrrhiza glabra*.

6.6. Screening of Antibacterial activity of *Curcuma longa* and *Glycyrrhiza glabra*:

Antibacterial activity of ethanolic extract of *Glycyrrhiza glabra*: Antibacterial activity of ethanolic extract of *Glycyrrhiza glabra* against *Paenibacillus popilliae* was observed by agar well diffusion. Results of antibacterial activity was observed as 8, 12, 15 and 17 mm zone of inhibition at concentration 1, 2, 3 and 4 mg/ml against *Paenibacillus popilliae*. Ciprofloxacin antibiotic used as positive control showed 30 mm zone of inhibition and negative control used as ethanol showed no clear zone of

inhibition against *Paenibacillus popilliae* **Plate (A)**. Antibacterial activity of aqueous *Glycyrrhiza glabra* extract was showed 10, 13, 17 and 21mm Zone of inhibition against *Paenibacillus popilliae*. Positive control and negative control was also used **Plate (B)**.



a) Plate A **b) Plate B**

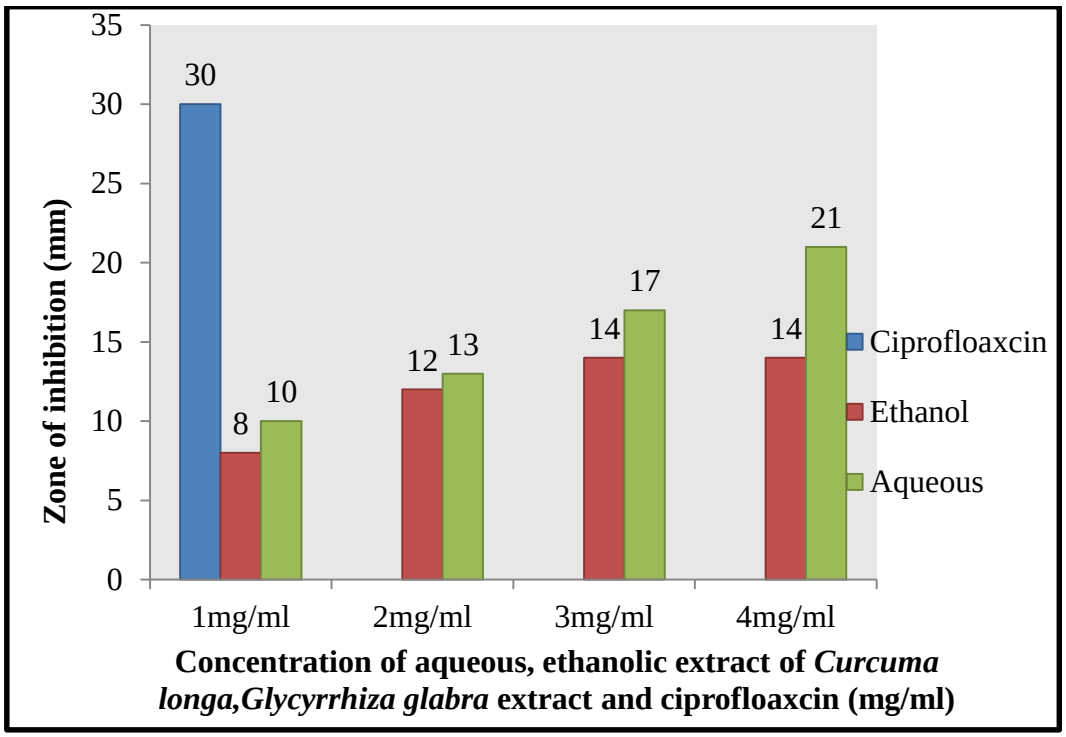
Figure 24: Results of antibacterial activity of ethanolic and aqueous extract of *Glycyrrhiza glabra*; a) Plate A and b) Plate B.

Table 9: Antibacterial activity of ethanolic and aqueous extract of *Glycyrrhiza glabra.*

Concentration (mg/ml)			**Zone of inhibition (mm)**			
Glycyrrhiza glabra		**Positive control**	**Ethanolic extract**	**Aqueous extract**	**Ciprofloaxcin***	**Ethanol/ Aqueous**
Ethanolic extract	**Aqueous extract**	**Ciprofloxacin**				
1	1	1	8±0.0	10±0.3	30±0.3	-
2	2	1	12±0.6	13±0.0	30±0.3	-
3	3	1	15±0.3	17±0.0	30±0.3	-
4	4	1	17±0.3	21±0.6	30 ± 0.3	-

(-) negative sign shows no clear zone of inhibition and (*) sign indicate the positive control of antibiotic ciprofloaxin. Diameter of Zone of inhibition is equal to the diameter of well (6mm) values are mean. All values are expressed as mean ± standard deviation of triplicates.

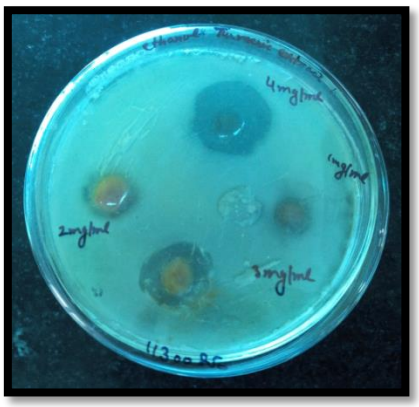
Table 9 showed antibacterial activity of ethanolic and aqueous *Glycyrrhiza glabra* extract results by agar well diffusion method at concentration of 1, 2, 3 and 4 in milligram per millilitre. 1 mg/ml concentration of Ciprofloxacin was used as standard positive control and ethanol, water used as negative control.



Graph 2: Antibacterial activity of ethanolic and aqueous extract of *Glycyrrhiza glabra*.

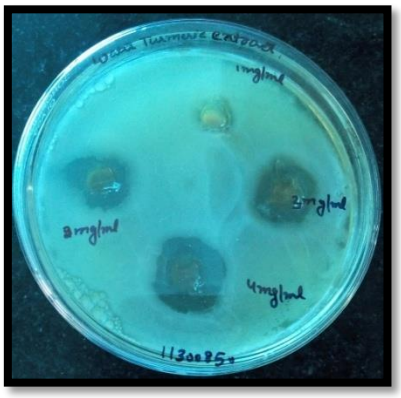
Graph 2: y- axis and x-axis of graph showed zone of inhibition in millimetre and concentration of ethanol, water extract of *Glycyrrhiza glabra* one milligram per millilitre. Ciprofloxacin used as a positive control in concentration of one milligram per millilitre.

Antibacterial activity of ethanolic and aqueous extract of *Curcuma longa*: The results of antibacterial activity of ethanolic extract of *Curcuma longa* at concentration of 1, 2, 3, 4 and 5mg/ml showed 9, 13 15, 22 and 24 mm zone of inhibition against *Paenibacillus popilliae* **Plate (C & D).** The results of aqueous extract of *Curcuma longa* at concentration of 1, 2, 3, 4 and 5 mg/ml showed 8, 12, 15, 18 and 19mm zone of inhibition. 1 mg/ml concentration of Ciprofloxacin showed 30mm zone of inhibition against *Paenibacillus popilliae* and negative control used as distilled water showed no clear zone of inhibition **Plate (E & F)**.

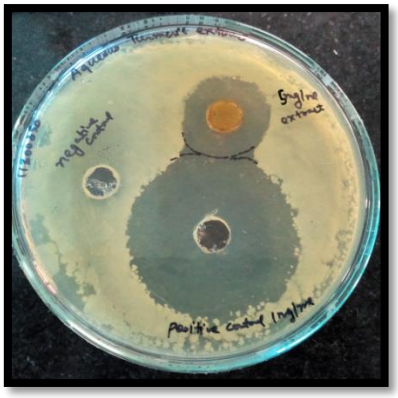


a) Plate C

b) Plate D



c) Plate E



d) Plate F

Figure 25: Results of antibacterial activity ethanolic and aqueous extract of *Curcuma longa* ; a) Plate C, b) Plate D, c) Plate E and d) Plate F.

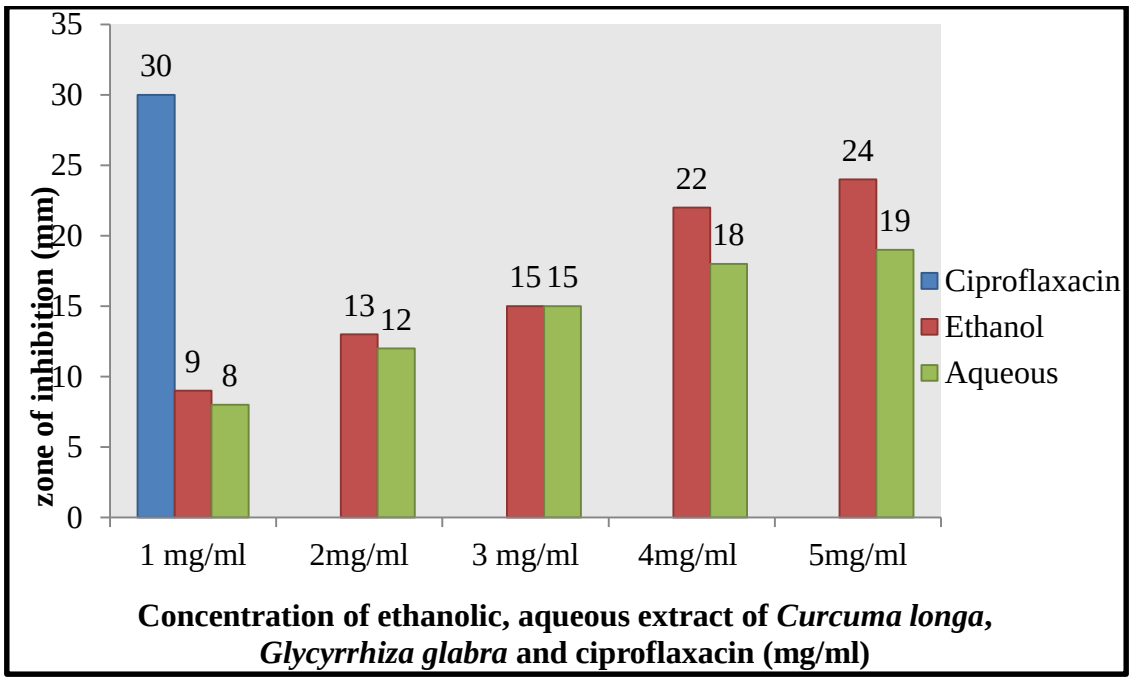
Table 10: Antibacterial activity of ethanolic and aqueous extract of *Curcuma longa*

Concentration (mg/ml)			**Zone of inhibition (mm)**			
Curcuma longa		**Control**	**Ethanolic extract**	**Aqueous extract**	**Control**	**Ethanol / Aqueous**
Ethanolic extract	**Aqueous extract**	**Ciprofloxacin ***				
1	**1**	**1**	9±0.6	8±0.3	30±0.3	-
2	**2**	**1**	13±0.6	12±0.6	30±0.3	-
3	**3**	**1**	15±0.6	15±0.3	30±0.3	-
4	**4**	**1**	22±0.0	18±0.3	30±0.3	-
5	**5**	**1**	24±0.6	19±0.6	30±0.3	-

(-) Negative sign shows no clear zone of inhibition of negative control and (*) sign showed positive control of antibiotic that is ciprofloxacin. Diameter of Zone of

inhibition is equal to the diameter of well (6mm) values are mean. All values are expressed as mean ± standard deviation of triplicates.

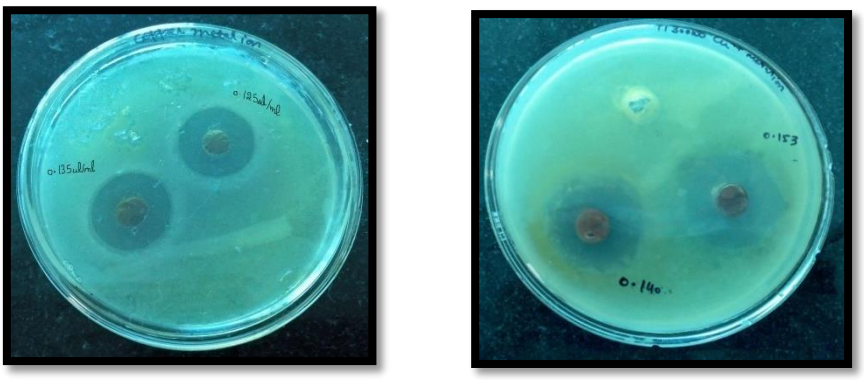
Table 10: The results of antibacterial activity of ethanolic and aqueous extract of *Curcuma longa* results at concentration of 1, 2, 3, 4 and 5 milligram per millilitre was shown 9, 13, 15, 22 and 24 millimetre zone of inhibition and 8, 12, 15, 18 and 19 in aqueous extract of *Curcuma longa* against *Paenibacillus popilliae*.



Graph 3: Antibacterial activity of ethanolic and aqueous extract of *Curcuma longa*.

Graph 3: y- axis and x- axis of graph showed zone of inhibition in millimetre and concentration of ethanol, aqueous extract of *Curcuma longa* in one milligram per millilitre. Ciprofloxacin used as a positive control at concentration of one milligram per millilitre.

Antibacterial activity of Copper metal ions against food spoilage bacteria: Antibacterial activity of the copper metal ion concentration 0.125, 0.13, 0.140 and 0.153µl/ml showed 17, 18, 20 and 21mm zone of inhibition against *Paenibacillus popilliae* **Plate (G).** Negative control used as distilled water was evaluated no clear zone *Paenibacillus popilliae* **Plate (H).**



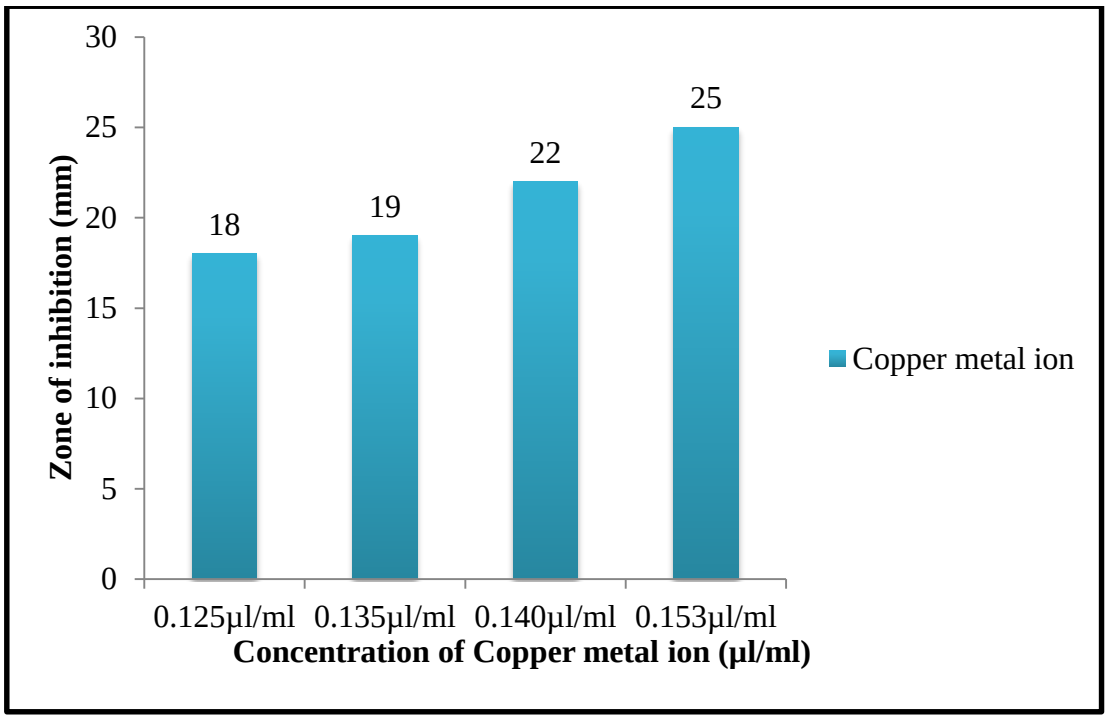
a) Plate G **b) Plate H**

Figure 26: Results of antibacterial activity of Copper metal ions; a) Plate G and b) Plate H.

Table 11: Antibacterial activity of Copper metal ion (Cu^{2+}) against *Paenibacillus popilliae.*

Concentration of copper metal ions (µl/ml)	**Zone of inhibition (mm)**	
	Copper metal ion	**Distilled water**
0.125	18±0.6	-
0.135	19±0.0	-
0.140	22±0.6	-
0.153	25±0.0	-

(-) negative control distilled water represented no zone of inhibition**.** Diameter of Zone of inhibition is equal to the diameter of well (6mm) values are mean. All values are expressed as mean ± standard deviation of triplicates. Table 11 shows the results of antibacterial activity of copper metal ion at concentration of 0.125, 0.135, 0.140 and 0.155 micro litre per millilitre was showed 18, 19, 22 and 25 millimetre zone of inhibition against *Paenibacillus popilliae*. Negative used as distilled water was showed no clear zone against *Paenibacillus popilliae*.

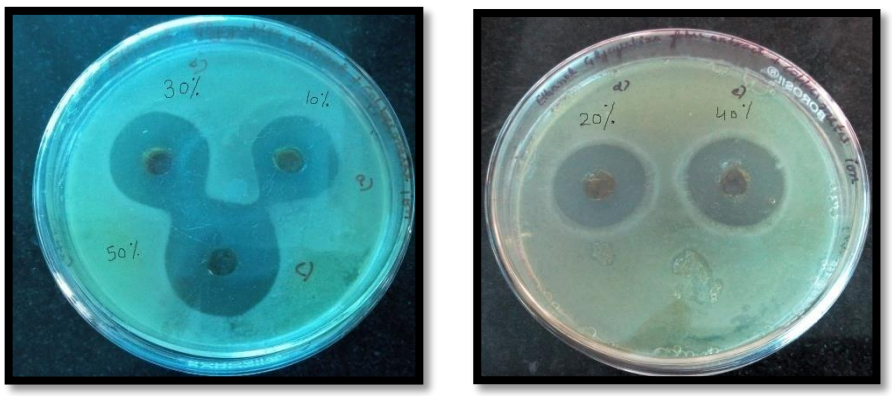


Graph 4: Antibacterial activity of copper metal ions against *Paenibacillus popilliae*.

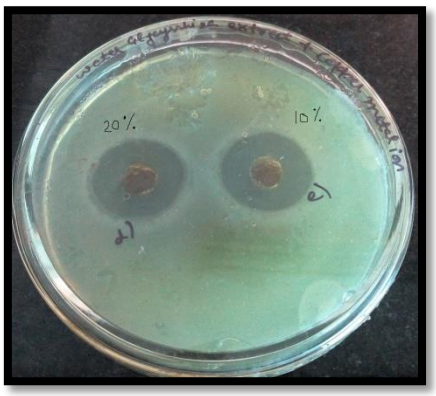
Graph 4: y- axis showed zone of inhibition in millimetre and x-axis showed the concentration of copper metal ions at 0.125µ/ml, 0.135µl/ml, 0.140µl/ml and 0.153 in micro litre per millilitre against *Paenibacillus popilliae*.

Combinatorial antibacterial activity of ethanolic and aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* with copper metal ion:

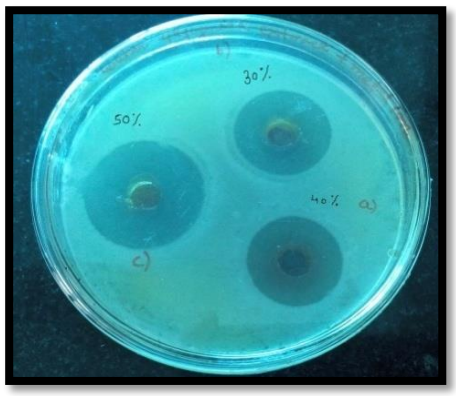
The results of combinatorial antibacterial of *Curcuma longa* and *Glycyrrhiza glabra* extract with copper metal ions at different concentration of 10%, 20%, 30%, 40% and 50% against *Paenibacillus popilliae* was evaluated by agar well diffusion method **Plate (I to P).**



a) Plate I **b) Plate J**

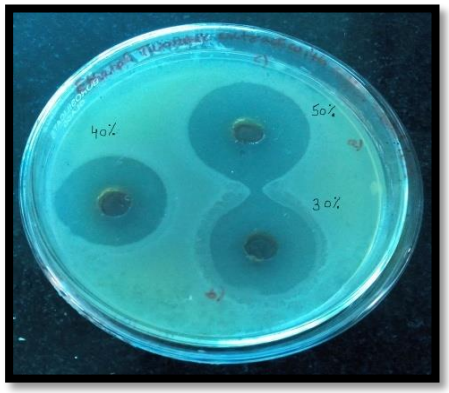


c) Plate K

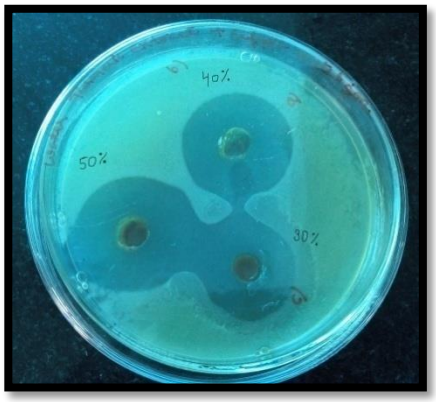


d) Plate L

e) Plate M



f) Plate N



g) Plate O

h) Plate P

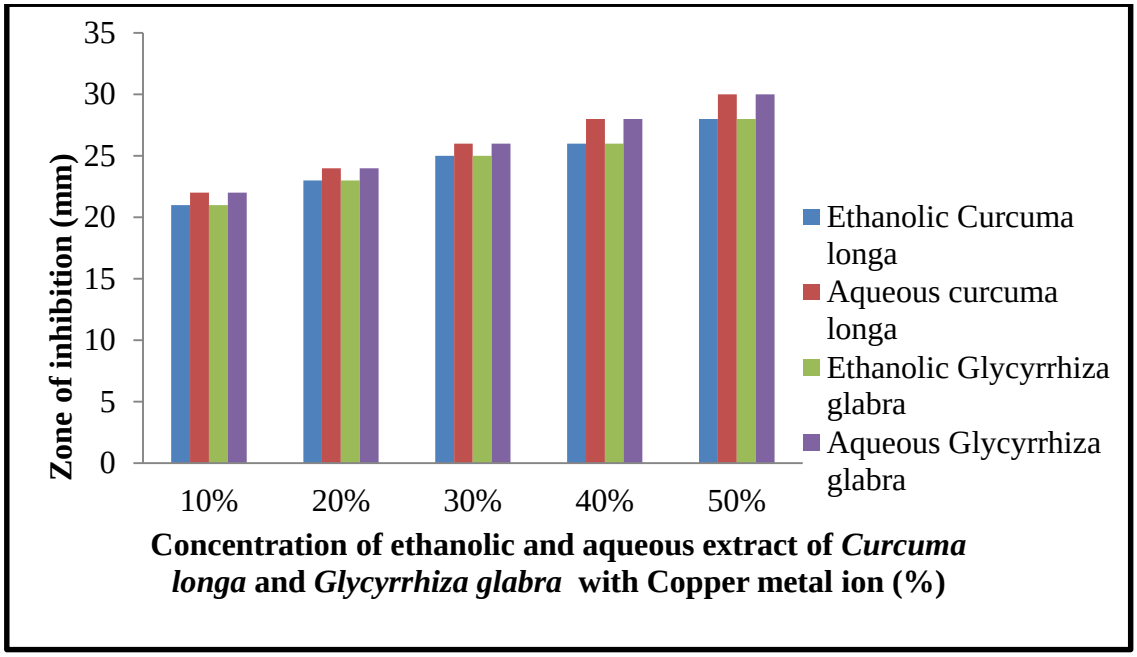
Figure 27: Results of combinatorial antibacterial activity of ethanolic and aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* with copper metal ions; a) Plate I, b) Plate J, c) Plate K , d) Plate L, e) Plate M, f) Plate N, g) Plate O and h) Plate P.

Table 12: Combinatorial antibacterial activity results of ethanolic and aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* with copper metal ion against *Paenibacillus popilliae*.

Concentration of plant extract with copper metal ions (%)	**Zone of inhibition (mm)**			
	Curcuma longa		***Glycyrrhiza glabra***	
	Ethanolic extract	**Aqueous extract**	**Ethanolic extract**	**Aqueous extract**
10	21±0.0	23±0.3	21±0.0	22±0.3
20	23±0.0	24±0.6	23±0.6	24±0.0
30	25±0.0	26±0.3	25±0.6	26±0.3
40	26±0.6	28±0.0	26±0.6	28±0.0
50	28±0.3	30±0.3	28±0.6	30±0.0

All values are expressed as mean ± standard deviation of triplicates.

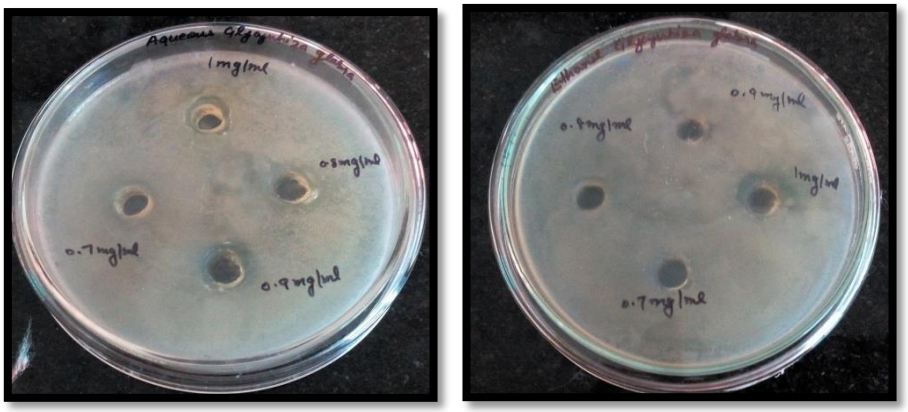
Table 12 showed the concentration of ethanolic extract of *Glycyrrhiza glabra* and *Curcuma longa* with copper metal ion at 10%, 20%, 30%, 40% and 50% was shown 21, 23, 25, 26 and 28 mm and 21, 23, 25, 26 and 28 mm zone of inhibition against *Paenibacillus popilliae*. Whereas the Concentration of aqueous extract of *Glycyrrhiza glabra* and *Curcuma longa* with copper metal ion at 10%, 20%, 30%, 40% and 50% was shown 23, 24, 26, 28 and 30 mm and 22, 24, 26, 28 and 30 mm zone of inhibition against *Paenibacillus popilliae.*



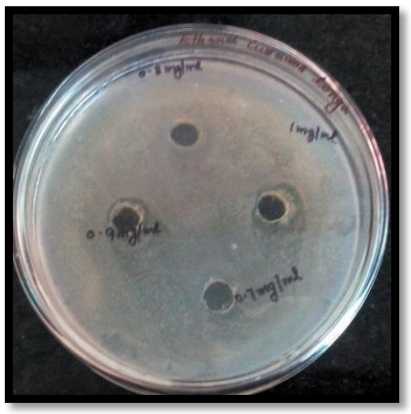
Graph 5: Combinatorial activity of ethanolic and aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* with Copper metal ion.

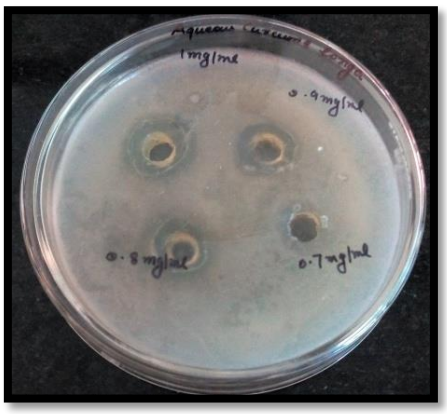
Graph 5: y-axis showed Zone of inhibition in millimetre (mm) and x- axis showed concentration of ethanolic and aqueous extract of *Glycyrrhiza glabra* extract with copper metal ion at 10, 20, 30, 40 and 50 in percentage (%).

6.7. Minimum inhibitory concentration of ethanolic and aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra*: Plate (Q) and Plate (R) showed the minimum inhibitory concentration of ethanolic and aqueous extract of *Glycyrrhiza glabra*. **Plate (S) and Plate (T)** showed the minimum inhibitory concentration of ethanolic and aqueous extract of *Curcuma longa* against *Paenibacillus popilliae.*



a) Plate Q **b) Plate R**





c) Plate S **d) Plate T**

Figure 28: Results of minimum inhibitory concentration of ethanolic and aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra*; a) Plate Q, b) Plate R, c) Plate S and d) Plate T.

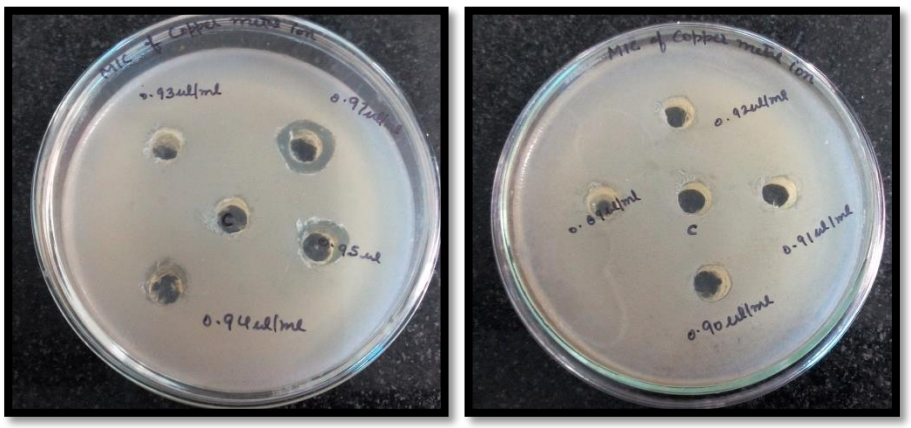
Table 13: Minimum inhibitory concentration results of ethanolic and aqueous extract *Curcuma longa* and *Glycyrrhiza glabra* against *Paenibacillus popilliae.*

Minimum inhibitory Concentration (mg/ml)	Zone of inhibition (mm)			
	Curcuma longa		*Glycyrrhiza glabra*	
	Ethanolic extract	Aqueous extract	Ethanolic extract	Aqueous extract
1	8±0.6	8±0.3	8±0.3	10±0.3
0.9	7±0.3	7±0.6	ND	8±0.6
0.8	ND	6±0.3	ND	7±0.6
0.7	ND	ND	ND	ND

ND: Not detected; All values are expressed as mean ± standard deviation of triplicates.

Table 13: The results of minimum inhibitory concentration at 0.9mg/ml of ethanolic extract of *Curcuma longa* showed 7 mm zone of inhibition and aqueous extract at 0.8mg/ml showed 6 mm zone of diameter. Whereas 1 mg/ml of minimum inhibitory concentration of ethanolic extract of *Glycyrrhiza glabra* showed 8 mm and aqueous extract at 0.8mg/ml showed 7 mm zone of inhibition against *Paenibacillus popilliae.*

6.8. Minimum inhibitory concentration of copper metal ion against *Paenibacillus popilliae*: Plate (U) and Plate (V) showed the minimum inhibitory concentration of copper metal ions from 0.97µ/ml to 0.90µl/ml and C denoted as a control (distilled water).



a) Plate U **b) Plate V**

Figure 29: Results of minimum inhibitory concentration of copper metal ion; a) Plate U and b) Plate V.

Table 14: Minimum inhibitory concentration of copper metal ions against *Paenibacillus popilliae*.

Concentration of copper metal ions (µl/ml)	Zone of inhibition (mm)	
	Copper metal ion	Control
0.97	9±0.6	ND
0.95	8±0.6	ND
0.94	6±0.6	ND
0.93	ND	ND
0.92	ND	ND
0.91	ND	ND
0.90	ND	ND

ND: Not detected; All values are expressed as mean ± standard deviation of triplicates.

Table 14: The result of minimum inhibitory concentration of copper metal ion was obtained at 0.94µl/mg against *Paenibacillus popilliae*. Distilled water was used as control.

6.9. Molecular characterization of Bacteria:

16s rRNA sequencing characterized the class of *Bacilli*, belong to the family of *Paenibacillaceae* and genus *Paenibacillus popilliae* was obtained. The results for identification of *Paenibacillus popilliae* isolated bacteria by 16s rRNA gene based phylogenetic analysis are shown in Fig 23. *Paenibacillus popilliae* are 33% closely related to *Paenibacillus apiaries* species.

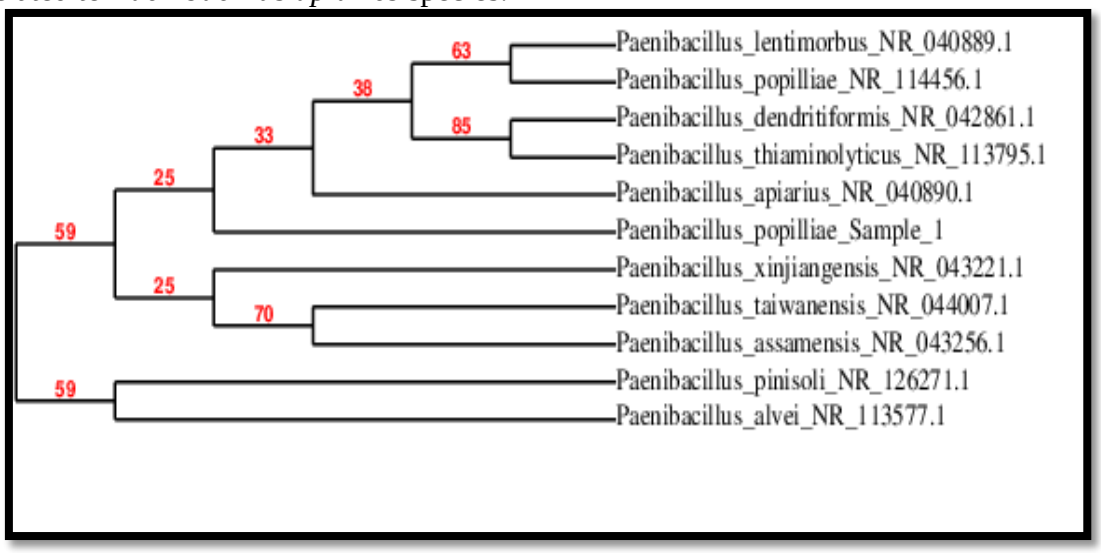
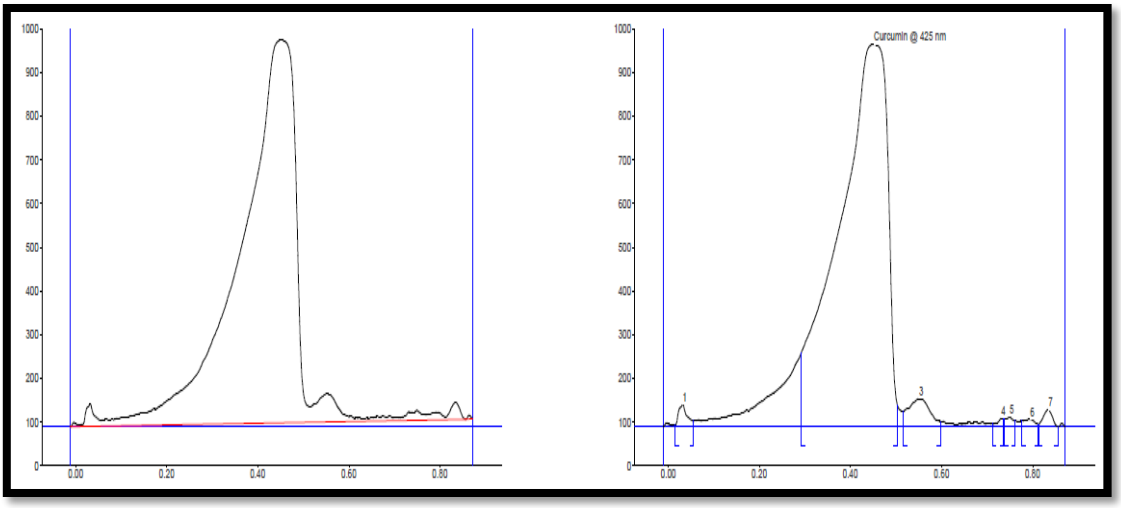


Figure 30: Phylogenetic tree results of isolated bacteria by 16s rRNA sequencing.

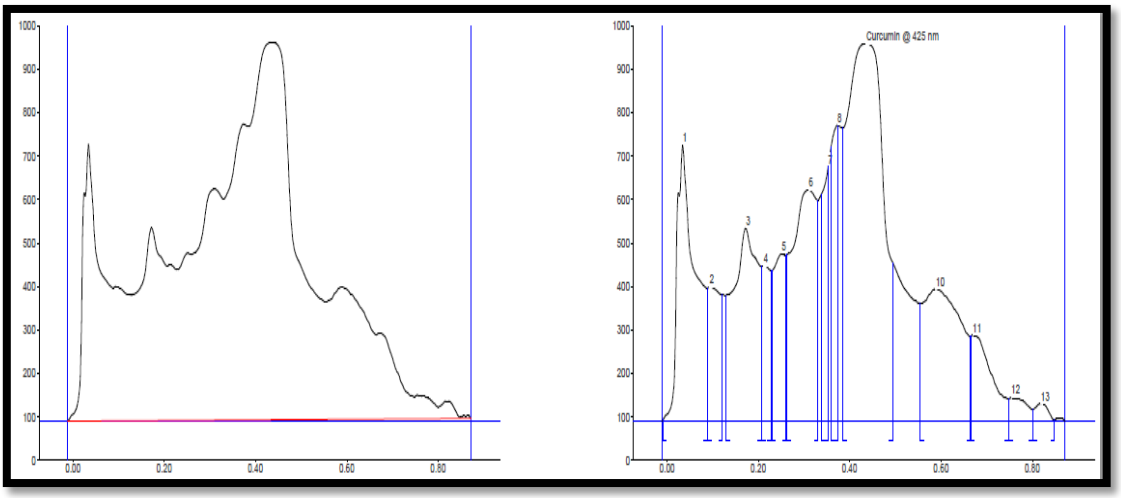
Forward Sequence:
AACCTAGGAGATGATGCTAGTGTTAGAGGTTTCGCAGTACCTTTGGTGCT
CGCAAGCTATAACACATTAAGCACTTCCGCCTGGGGAGTACGGTCGCAAG
ACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTC
TGACCGTCCTAAAAATAGGGCTTCCCTTCGGGGCAAAAGTGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTAACTTTAGTTGCCAGCATTGAGTTGGGCACTCTAAA
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
ATGCCCCTTATGACCTGGGCTACACACGTACTACAATGGCTGGAACAACG
GGAAGCGAAGCCGCGAGGTGGAGCGAATCCTAAAAAGCCGTTCTCAGTTC
GGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATTGCTAGTAATCG
CGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCC
CGTCACACCACGAGAGTTTACAACACCCGAAGTCGGTGAGGTAACCTTTT
GGAGCCAGCCGCCGAAGGTGGGATAGATGATTGGGGTGAAGTCGTACAGG
TAACCCTAAAA

Figure 31: 16s rRNA gene sequences of isolated bacteria (*Paenibacillus popilliae*).

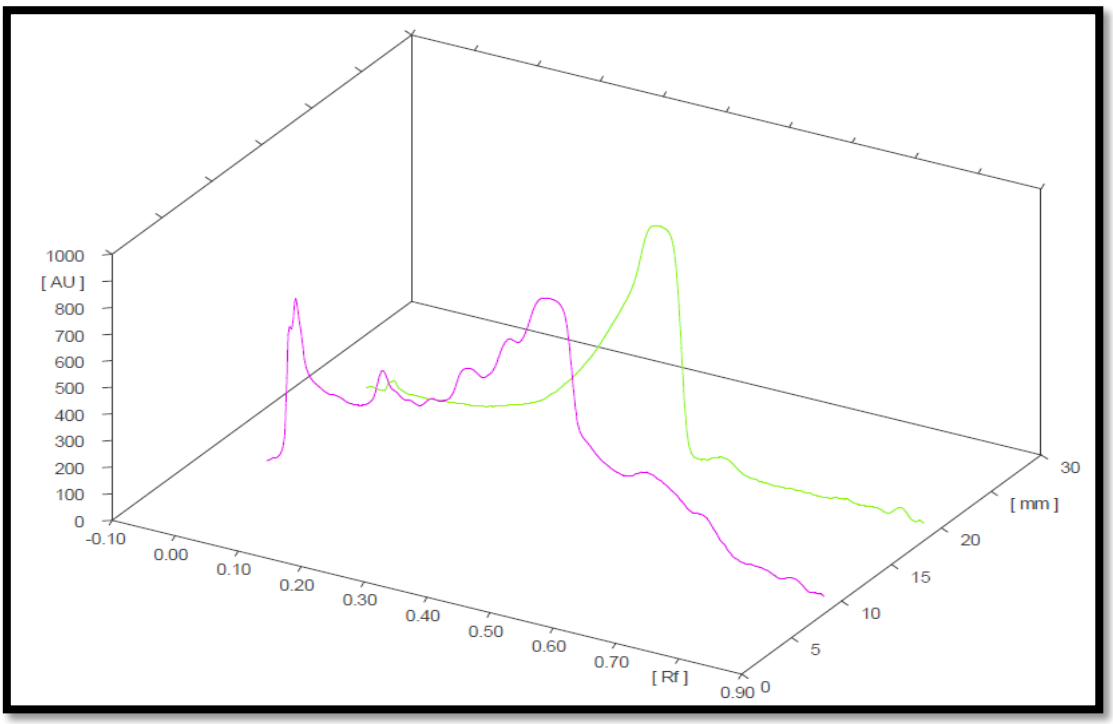
7.0. Estimation of Curcumin content in *Curcuma longa* rhizome extract: Table 15 shows that the curcumin sample was observed at Rf value from 0.29 to 0.50 in the chromatogram of the sample extracted from rhizomes of *Curcuma longa*. For the determination of Curcumin content, curves of area vs. concentration peak results was analysed and calculated as 10.37%.



a) HPTLC results showing absorbance of Curcumin compound in *Curcuma longa* at 425 nm taken as standard



b) HPTLC chromatogram showing absorbance of curcumin sample at 425nm.



c) Superimposed spectra showing absorbance of curcumin standard (Green Curve) and sample (Pink Curve) at 425 wavelength.

Figure 32: HPTLC peak results; a) HPTLC results showing absorbance of Curcumin compound in *Curcuma longa* at 425 nm taken as standard; b) HPTLC chromatogram showing absorbance of curcumin sample at 425nm;c) Superimposed spectra showing absorbance of curcumin standard (Green Curve) and sample (Pink Curve) at 425 wavelength.

Table 15 showing Rf values by HPTLC for determination of Curcumin, demethoxycurcumin and bisdemethoxycurcumin (Paramasivam *et al.*, 2008).

Peak	Start Rf	Start height	Max Rf	End Rf	Area %	Compound
4	0.21	356.4	0.21	0.23	2.88	Bisdemethoxycurcumin
8m	0.36	632.4	0.37	0.37	4.13	demethoxycurcumin
9m	0.39	675.4	0.44	0.49	30.04	Curcumin

In this study, *Curcuma longa* and *Glycyrrhiza glabra* extract has shown significant activity against food spoilage bacteria. *Paenibacillus popilliae* was isolated and

confirmed by biochemical testing and culturing it on selective media and 16s rRNA sequencing analysis

As per the present study naturally occurring antimicrobial compounds were responsible for antimicrobial activity. Steroid compound was found in all aqueous and ethanolic extract of *Glycyrrhiza glabra* and *Curcuma longa* as compare to the other compounds.

The results of antibacterial activity of aqueous extract of *Glycyrrhiza glabra* was showed 10mm, 13mm 17mm and 21mm zone of inhibition against *Paenibacillus popilliae* as compare to ethanolic extract of *Glycyrrhiza glabra*. The results of antibacterial activity of ethanolic extract of *Curcuma longa* was showed 9mm, 13mm, 15mm, 22mm and 24mm zone of inhibition against *Paenibacillus popilliae* as compare to the aqueous extract of *Curcuma longa*. Copper metal ion showed 18mm, 19mm, 22mm and 25mm maximum zone of inhibition against *Paenibacillus popilliae* as compare to individual aqueous and ethanol plant extract.

Copper metal ion was found best elicitors selected in the study to enhance the activity of plant extract. The activity of plant extracts were increased by increase in concentration of copper metal ions about 10%, 20%, 30%, 40% and 50%. The results of combinatorial activity of aqueous extract of *Glycyrrhiza glabra* with copper metal ion was showed 22mm, 24mm, 26mm, 28mm and 30 mm maximum zone of inhibition against *Paenibacillus popilliae* as compare to the ethanolic extract of *Glycyrrhiza glabra*. Whereas the combinatorial activity of aqueous extract of *Curcuma longa* along with copper metal ion was shown 23mm, 24mm, 26mm, 28mm and 30 mm zone of inhibition against *Paenibacillus popilliae* as compare to ethanolic extract.

Minimum inhibitory concentration results of aqueous root extract of *Curcuma longa* and *Glycyrrhiza glabra* showed least MIC value at 0.8 mg/ml against *Paenibacillus popilliae*. Where as in case of copper metal ion showed least MIC value at 0.94µl/mg against *Paenibacillus popilliae*.

For the identification of Curcumin, demethoxycurcumin and bis-demethoxycurcumin in rhizome of *Curcuma longa* were confirmed by the superimposing UV spectra of sample and standards with Rf values are 0.44, 0.37 and 0.21 at 425nm wavelength.

CHAPTER 7

Conclusion and Future scope

The result of present study concluded, that the effect of elicitors on antibacterial activity of various extracts has been reported that aqueous extract of plants showed maximum activity in conjugation with copper metal ions against food spoilage bacteria as compared to ethanolic extract of plants. Aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* has been showed greater affinity to bind with copper metal ion and enhance the antimicrobial activity to inhibit the growth of food spoilage bacteria.

Curcumin is active compound present in the extract of *Curcuma longa*, which showed maximum antibacterial property against food spoilage bacteria. On other hand, Glycyrrhizic acid is act as active compound responsible for antibacterial activity against food spoilage bacteria. These natural compounds are used in the food industry to overcome the problems of contamination from microorganisms. Shelf life of food can be increased by adding these natural compounds to food products.

Attempted study is used to increase the antibacterial activity of *Curcuma longa* and *Glycyrrhiza glabra extract* by addition of copper metal ion to crude extract at different concentration. The result was obtained that the aqueous extract of *Curcuma longa* and *Glycyrrhiza glabra* showed maximum zone of inhibition against food spoilage bacteria in the presence of copper metal ions. The potency and efficacy of plant extract with metal ion promote good quality control of food products and provide the safety for consumers from toxicity.

To overcome several problems generated by food spoilage bacteria, antimicrobial compounds of *Curcuma longa* and *Glycyrrhiza glabra* extract provides a great opportunity in the field of natural product chemistry, Pharmacognosy, pharmacology and other field of life sciences. Work was done in the direction to getting the natural compounds from plants for better mankind.

The importance of natural products in the future is drug discovery; natural compounds will continue to serves as active compounds for drug development. Traditional knowledge and database provide new functional compounds to reduce money, time and more important toxicity-three main parameter handles in drug development.

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APPENDIX

Reagents Used In the Research Work

Reagents used for Bacteriology Procedure

1. Nutrient Agar Media:

S.NO.	Nutrient Agar Media	Grams/litre
1.	Peptic digest of animal tissue	5.0
2.	Sodium Chloride	5.0
3.	Beef extract	1.5
4.	Yeast extract	1.5
5.	Agar	15.0
6.	Final pH (at 25° C)	7.4±0.2

For 1000ml media preparation, suspend 28gm of media in 1000ml distilled water and shake well to dissolve. Heat it gently to dissolve properly. Now sterilize it by Autoclaving at 121°C at 15psi for 15 minutes.

2. Nutrient Broth Media:

S.NO.	Nutrient Broth Media	Grams/litre
1.	Peptic digest of animal tissue	5.0
2.	Sodium Chloride	5.0
3.	Beef extract	1.5
4.	Yeast extract	1.5
5.	Final pH (at 25° C)	7.4±0.2

For 1000ml media preparation, suspend 13gm of media in 1000ml distilled water and shake well to dissolve. Heat it gently to dissolve properly. Now sterilize it by Autoclaving at 121°C at 15psi for 15 minutes

3.Simmon Citrate Agar Media:

S.NO.	Simmon Citrate agar Media	Grams/litre
1.	Agar	15.0
2.	Sodium Chloride	5.0
3.	Sodium citrate	2.0
4.	Dipotassium phosphate	1.0

For 1000ml media preparation, suspend 24.28gm of media in 1000ml distilled water and shake well to dissolve. Heat it gently to dissolve properly. Now sterilize it by Autoclaving at 121°C at 15psi for 15 minutes. Avoid overheating. Cool to 45-45°C.

4.Urea Broth:

S.NO	Urea Broth	Gram/litre
1.	Urea	2.0
2.	Dipotassium	9.50
3.	Monophosphate	9.1
4.	Yeast extract	0.10
5.	Phenol Red	0.01
6.	pH	6.8±0.25
7.	Distilled Water	1 litre

For 1000ml media preparation, suspend 38.7gm of media in 1000ml distilled water and shake well to dissolve. Heat it gently to dissolve properly. Now sterilize it by Autoclaving at 121°C at 15psi for 15 minutes. Avoid overheating. Cool to 45-45°C.

5.Tryptone Broth:

S.No.	Tryptone Broth Media	Grams/litre
1.	Tryptone	20.0
2.	Sodium chloride	5.0
3.	Dextrose	1.0
4.	pH (25°C)	7.3±0.2

For 1000ml media preparation, suspend 26gm of media in 1000ml distilled water and shake well to dissolve. Heat it gently to dissolve properly. Now sterilize it by Autoclaving at 121°C at 15psi for 15 minutes.

6.Peptone Water Broth:

S.No	Peptone Water Broth	Percentage (%)
1.	Enzymatic pH 20% Solution	6.5-7.5%
2.	Moisture	Max. 6%
3.	Total nitrogen	12%w/w

For 1000ml media preparation, suspend 16.09 gm of media in 1000ml distilled water and shake well to dissolve. Heat it gently to dissolve properly. Now sterilize it by Autoclaving at 121°C at 15psi for 15 minutes.

7. Methyl red-Vogesproskaeur Agar:

S.NO.	Methyl red-vogesproskaeur Agar	Grams/litre
1.	Buffered Peptone	7.0
2.	Dextrose	5.0
3.	Dipotassium phosphate	5.0
4.	Final pH (25° C)	6.9±0.2

17.0gm in 1000ml of distilled water heat if nessary dissolve the media.Inoculate in culture and incubate at specified temperature. Now sterilize it by Autoclaving at 121°C at 15psi for 15 minutes.

7. EMB Agar Media:

S.NO.	EMB Agar Media	Gram/ litre
1.	Agar	13.5
2.	Di-potassium phosphate	2
3.	Eosin-Y	0.4
4.	Lactose	5
5.	Methylene Blue	0.065
6.	Peptic digest of animal tissue	10
7.	Sucrose	5
8.	Final pH (25°C)	7.3±0.2

For the preparation of 1000ml media, 35.9gm of media dissolve in 1000ml distilled water and shake well to dissolve. Heat it gently to dissolve properly. Now sterilize it by Autoclaving at 121°C at 15psi for 15 minutes.

9. Copper Metal Powder :

S.NO.	Copper metal powder	Percentage (%)
1.	Nitric acid insoluble matter	0.05
2.	Antimony	0.005
3.	Arsenic	0.0002
4.	Iron	0.005
5.	Lead	0.05
6.	Manganese	0.005
7.	Silver	0.005
8.	Minimum assay	99