



**Evaluation and characterization of plant metabolites complex as  
an effective microbicidal agent and bio preservatives.**

**Project Report**

Submitted in partial fulfilment of the requirements for the degree of

Master of Technology

Biotechnology

Submitted by

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## CERTIFICATE

This is to certify that **Poornesh G (11502522)** have completed the project entitled “*Evaluation and characterization of plant metabolites complex as an effective microbicidal agent and bio preservatives*” 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 in biotechnology.

Date:

Supervisor Signature:

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## DECLARATION STATEMENT

We hereby declare that the project entitled “*Evaluation and characterization of plant metabolites complex as an effective microbicidal agent and bio preservatives*” is an authentic record of our own work carried out at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, for the partial fulfilment of the award of Master of Technology in Biotechnology under the guidance of *Mr. Amarish Kumar Sharma*

This work is our original work and has not been submitted for any degree/diploma in this or any other University. The information furnished in this report is genuine to the best of my knowledge and belief.

Place:

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## TABLE OF CONTENTS

<b>CHAPTERS</b>	<b>TITLE</b>	<b>PAGE NO.</b>
	<b>ABSTRACT</b>	i
	<b>LIST OF TABLES</b>	ii
	<b>LIST OF FIGURES</b>	iii
	<b>LIST OF GRAPHS</b>	iv
	<b>LIST OF ABBREVIATIONS</b>	v
	<b>LIST OF SYMBOLS</b>	vi
<b>1.</b>	<b>INTRODUCTION</b>	1
	1.1. Food Bio preservatives	1
	1.2. Random Screening	2
	1.3. Syzygium aromaticum	2
	1.3.1. Active compounds in clove	3
	1.3.2. Medicinal properties	3
	1.4. Cannabis L.	3
	1.4.1. Medicinal properties	4
	1.5. Tinospora cordifolia	4
<b>2.</b>	<b>TERMINOLOGY</b>	6
<b>3.</b>	<b>REVIEW OF LITERATURE</b>	7
<b>4.</b>	<b>RATIONALE AND SCOPE OF STUDY</b>	13
	4.1. Background of this study	13

	4.2. Statement of Problem	14
	4.3. A snapshot of this research study	16
<b>5.</b>	<b>OBJECTIVES OF THE STUDY</b>	<b>17</b>
<b>6.</b>	<b>MATERIALS AND METHODOLOGY</b>	<b>18</b>
	6.1. Equipment used	18
	6.2. Plant sample and microbial culture collection	18
	6.3. Preparation of Plant extracts	18
	6.3.1. Drying of plants	18
	6.3.2. Preparation of powder	19
	6.3.3. Usage of solvents for extraction	19
	6.3.4. Soxhlet apparatus	
	6.4. Antimicrobial activity	19
	6.4.1. Preparation of bacterial culture	19
	6.4.1.1. E. coli culture	19
	6.4.1.2. S. aureus culture	20
	6.4.2. Well diffusion assay	20
<b>7.</b>	<b>RESULTS</b>	<b>21</b>
	7.1. Plant materials for drying	21
	7.2. Soxhlet extraction	22
	7.3. Antibacterial activity	23
	7.4. Screening of bioactive compounds	24

	7.5 ZOI of Ethanolic extracts of plants	24
	7.6. ZOI of Methanolic extracts of plants	25
	7.7. ZOI of Acetone extracts of plants	26
	7.8. ZOI of Chloroform extracts of plants	27
	7.9. Comparison of all plant extracts	28
<b>8.</b>	<b>EXPERIMENTAL WORK</b>	<b>29</b>
	8.1. Gas Chromatography-Mass Spectrometry	30
<b>9.</b>	<b>CONCLUSION AND FUTURE SCOPE</b>	<b>32</b>
<b>10.</b>	<b>REFERENCES</b>	<b>33</b>

## **ABSTRACT**

Food products requires protection from spoilage during their preparation, storage and distribution, which gives desired shelf life. The problem of spoilage of food products can be solved with the development of food preservatives. Food preservatives helps in preservation of food because these can fight continuously against microorganisms, which are responsible for food spoilage and makes the food unsafe. The current research work is to study the type of microorganisms which normally spoils the food products and to find the antimicrobial solutions by natural means with the help of explants such as *Syzygium aromaticum*, *Cannabis L.* and *Tinospora cordifolia*. The ethanolic and methanolic extracts of the plants were used for examining the antimicrobial properties. On the basis of observation of results, the extracts of two different solvents were coupled with the essential metal ions. The best results of zone of inhibitions were optimized for the development of bio preservatives. The methanolic extract of clove with the metal ion  $Fe^{2+}$  at the concentration of 0.5g/mL and 1g/5mL shows the high degree of resistance against the food spoiling microorganisms. Also, the interaction and quality of the extract and metal ion couple has been studied through Gas chromatography-Mass spectrometry to produce a biopreservative.

### **KEY WORDS:**

Antimicrobial properties, ethanolic and methanolic extracts, zone of inhibition, metal ions.



## LIST OF TABLES

<b>Table No.</b>	<b>Name</b>	<b>Page no.</b>
1.	Botanical classification of <i>Syzygium aromaticum</i>	2
2.	Botanical classification of <i>Cannabis L.</i>	4
3.	Botanical classification of <i>Tinospora cordifolia</i>	5
4.	List of Plants materials and parts used	18
5.	List of solvents used for extraction	19
6.	Zone of inhibition of Ethanolic extracts of plants	24
7.	Zone of inhibition of Methanolic extracts of plants	25
8.	Zone of inhibition of Acetone extracts of plants	26
9.	Zone of inhibition of Chloroform extracts of plants	27
10.	Comparison of all plant extracts	28
11.	Methanolic extract of clove and Iron coupled	30
12.	Library of compounds GC-MS	31

## LIST OF FIGURES

<b>Figure No.</b>	<b>Name</b>	<b>Page no.</b>
1.	Drying of Cloves	21
2.	Drying of <i>Tinospora cordifolia</i>	21
3.	Drying of <i>Cannabis L.</i>	21
4.	Images for extraction experiment	22
5.	Antibacterial assay against <i>E. coli</i> and <i>S. aureus</i> .	23
6.	Antibacterial assay on Methanolic extract of clove-Fe <sup>2+</sup>	29
7.	Gas chromatography result for clove and Fe <sup>2+</sup>	31

## LIST OF GRAPHS

<b>Graph No.</b>	<b>Name</b>	<b>Page no.</b>
1.	Zone of inhibition of Ethanolic extract of plants	24
2.	Zone of inhibition of Methanolic extract of plants	25
3.	Zone of inhibition of Acetone extract of plants	26
4.	Zone of inhibition of Chloroform extract of plants	27
5.	Comparison of all plant extracts	28

## LIST OF ABBREVIATIONS

CLSI	Clinical and Laboratory Standards Institute
EC <sub>50</sub>	Half Maximal Effective Concentration
Fe	Iron
GC	Gas chromatography
IC <sub>50</sub>	50% Inhibitory Concentration
LB	Liquid broth (Luria Bertani -broth)
MS	Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
Mm	Millimeter
ZOI	Zone of Inhibition

## LIST OF SYMBOLS

%	-	Percentage
$\mu\text{l}$	-	Micro liter
$\times$	-	Multiplication
/	-	Division
$\wedge$	-	Power of
$^{\circ}\text{C}$	-	Degree Celsius
nm	-	Nanometer
ml	-	Milliliter

# 1. INTRODUCTION

## 1.1. Food Bio preservatives

Food products require safeguard from decay during their preparation, storage and distribution, which provide them desired shelf life. The requirement for prolonged shelf life of the food products extended due to the sold and transportation of food products in areas which are far distant from their production sites. This problem of spoilage of food products can be solved with the development of food preservative. Food preservative helps in preservation of food because these can fight continuously against microorganisms, which are responsible for food spoilage and makes the food unsafe. Heating, cooling and addition of antibacterial combinations can be used to reduce the risk of bursts of food poisoning. The major drawback of using these techniques is nutrients loss and change in characteristic of food. The need of new preservation techniques over traditional techniques is due to the amplified consumer call for tasty, nutritious, natural and easy-to-handle food products.

Traditional method of food preservation includes the use of organic acids, for example acetic, lactic, benzoic and sorbic acid. These organic acids hinder the growth of microorganism including their spore. Safety and stability are the major concern during the production of food products, there for proper measures are taken. In contemporary user trends and food legislation have made the successful realization of this independent much more of an encounter to the food industry. Initially, consumers require more high class, preservative-free, safe but mildly managed foods with prolonged shelf-life. For controlling the outgrowth of spore forming bacteria and fungus, acidity and sterilization treatments are two main factors applied for extending shelf life of food products. Addressing the need of consumers, calls for ground-breaking approaches to ensure preservation of products.

Secondly, regulation has limited the use and allowable levels of some currently accepted preservatives in various foods. An increasing number of consumers prefer negligibly processed foods, prepared without chemical preservatives. Current approaches are increasingly focussed towards possibilities offered by biological preservation by knowing the knowledge of the complexity of microbial interactions. Spices and herbs can also be used for alteration and increased taste, and also for improve shelf-life for their antimicrobial nature. Some of these same ingredients are also known to contribute to the self-defence of plants against contagious organisms.

## 1.2. Random Screening

The process of selecting the plants to get an complex that shows antimicrobial activity against the food spoiling microorganisms were chosen randomly with the help of research articles and traditional plants. The plants selected for this study is *Syzygium aromaticum*, *Cannabis L.*, and *Tinospora cordifolia*.

The choice of the plants were also given preference such that the publication is available in limited numbers. The detailed description of each plants are as follows:

## 1.3. *Syzygium aromaticum*

It's an effective wellbeing supplement with a wide assortment of employments and applications. From their antiquated excursion along the flavour course to their cutting edge use as a recuperating compound. The clove plant develops as a little, reduced, evergreen shrubbery, which flourishes in warm, muggy, atmospheres. On the off chance that permitted to blossom, the plant creates a striking pink bloom, which is then trailed by purple berries.

<b>Kingdom</b>	Plantae
<b>Order</b>	Myrtales
<b>Family</b>	Myrtaceae
<b>Genus</b>	<i>Syzygium</i>
<b>Species</b>	<i>Syzygium aromaticum</i>

**Table 1. Botanical Classification of *Syzygium aromaticum***

### 1.3.1. Main active compounds in clove:

The clove bud contains an uncommon blend of mixes found in no other plant, giving the herb its exceptional restorative properties. Cloves contain - among different mixes - gallotannins, triterpenes, flavonoids, and phenolic acids. Oil got from Cloves contains extra mixes including b-caryophyllene, eugenol, and eugenol acetic acid derivation.

### **1.3.2. Medicinal properties of cloves:**

Dietary home grown utilization of cloves can aid in the alleviation of a wide assortment of infections, both inner as well outer. As a supplement, the flavour can either be utilized as a part of its entire shape, ground frame, or lessened to its fundamental oil.

- Clove utilize can mitigate and unwind the inward covering of the insides, supporting in processing. It can likewise help in calming annoyed stomach.
- Cloves can help the throat deliver more mucus and go about as an expectorant, making hacks not so much serious but rather more gainful.
- Clove has been appeared to have pain relieving properties. This property is especially successful for tooth torment. Entire clove can be connected straightforwardly to the gum in issue zones. The hypersensitive film of the gums promptly assimilates oil from the clove, giving topical help from pain. Albeit less successful, clove can likewise be connected to external skin to help with sun blaze or toxic substance ivy.
- Clove can go about as an antimicrobial operator, slaughtering parasites and microbes in the stomach related tract. In fitting dose, it can assuage intemperate gas bloating.
- There is some confirmation that specific mixes in clove go about as antihistamines, keeping sinus paths clear and open.
- At the point when the oil is connected topically, it can calm agony from ailment, joint inflammation, or other irritation based torment.

### **1.4. Cannabis**

The utilization of cannabis as a solution has not been thoroughly experimentally tried, regularly because of generation confinements and other administrative regulations. There is restricted proof proposing cannabis can be utilized to diminish queasiness and heaving amid chemotherapy, to enhance hunger in individuals with HIV/AIDS, and to treat unending agony and muscle spasms. Its utilization for other restorative applications, be that as it may, is deficient for decisions about security or viability.



<b>Kingdom</b>	Plantae
<b>Order</b>	Rosales
<b>Family</b>	canabaceae
<b>Genus</b>	Cannabis L.

**Table 2. Botanical classification of Cannabis L.**

### **1.4.1. Medical importance of Cannabis L.**

- Fleeting use increments both minor and major antagonistic effects. Common reactions incorporate wooziness, feeling tired, heaving, and hallucinations.
- Long-term impacts of cannabis are not clear. Concerns including memory and comprehension issues, danger of compulsion, schizophrenia in youngsters, and the danger of kids taking it by accident.

### **1.5. Tinospora cordifolia**

T. cordifolia is a glabrous, juicy, ascending bush local to India furthermore found in Burma and Sri Lanka. It flourishes effortlessly in hot areas, frequently developing to awesome statures, and climbing the trunks of huge neem trees. The bark is dim or rich white, profoundly separated spirally and longitudinally, with extensive rosette-like lenticels. The wood is pale, delicate, permeable, and when naturally cut, rapidly accept a yellow shade. The twigs bear flat, heart-molded leaves, unisexual greenish blooms in summer, and red berries in winter. Long string like flying roots emerge out of the branches. The gooey sap is pale yellow, with a scent and a disgusting sharp taste.

<b>Kingdom</b>	Plantae
<b>Order</b>	Ranunculales
<b>Family</b>	Menispermaceae
<b>Genus</b>	Tinospora
<b>Species</b>	T. cordifolia

**Table 3. Botanical Classification of *Tinospora cordifolia***

**Medical Importance of *Tinospora cordifolia*:**

- *T. cordifolia* is utilized as a part of the Indian Ayurvedic arrangement of pharmaceutical for the treatment of jaundice, diabetes, and rheumatoid joint pain, and is additionally utilized as an immunostimulant.
- Tests have inspected its antineoplastic, cancer prevention agent, hepatoprotective, hypolipidemic, and immunologic properties; be that as it may, couple of clinical trials exist.

## **2. TERMINOLOGY**

### **2.1. Antibacterial activity:**

Anything that obliterates microscopic organisms or smothers their development or their capacity to repeat. Warm, chemicals, for example, chlorine, and anti-infection sedates all have antibacterial properties. Using several extracts from the plants are studied for such activity.

### **2.2. Minimum inhibitory concentration:**

It is a measure of potency shown by any metabolite act against the other. In the microbiological era, it determines and refers to activity of any substance that delimits the growth of an organism and creates an area limiting to its surrounding. The higher the formation of zone the higher the virulence of any metabolite is.

### **2.3. Soxhlet apparatus:**

An apparatus that is used for extraction principles. Here in this project, extraction of plant using solvents of polar and non-polar has been performed. The apparatus consists of heating mantle, distillation apparatus and condenser. The effective extracts are produced in setting up the optimum conditions. The boiling point temperature can be adjusted.

### **2.4. Polar and Non-polar Solvents:**

For the purpose of extraction, employment of polar and non-polar solvents has been preferred. The polar solvents used here are methanol and ethanol as well non-polar solvents used are acetone and chloroform. These solvents makes the extract out of plants to be formed as liquid solutions and later be concentrated.

### **2.5. Gas chromatography:**

The instrument used for performing purification and analyse the quality as well as the constituents of any analyte studied according to the retention time. The mobile phase is mixture of gases and stationary phase is the analyte.

### **2.6. Mass spectrometry:**

An analytical technique which used to study the composition of an analyte based on the mass to charge ratio.

### **3. REVIEW OF LITERATURE**

#### **3.1. Antimicrobials**

While trying to battle the different types of illness that have kept on plaguing people from time immemorial right up 'til the present time, diverse sorts of antimicrobials have created to battle the harmful microorganisms in charge of these maladies. Antimicrobials, are substances that kills, repress development of microorganism, could be in the type of anti-toxins, which are results of microbes or orchestrated subsidiaries (Cowan, 1999), antimicrobicidal peptides created by mixture of living beings and also a few microorganisms (Jenssen et al. 2006) and restorative plants, which seem, by all accounts, at the centre of standard solution nowadays (Cowan, 1999).

#### **3.2. Various classification and origin of antimicrobials**

Diverse sorts in antimicrobials exist: anti-infection agents, hostile to viral, against contagious, against protozoan and so forth. Anti-infection agents are utilized as a part of the action of bacterial diseases and may be acquired from either normal or manufactured source. These are from manufactured sources are sulphonamides and quinolones (Singh and Barrett, 2006). Most anti-infection agents apply their activity also by hindrance of the bacterial cell divider or protein union. Special cases are the quinolones that restrain Deoxyribonucleic acid amalgamation, and those sulphamethoxazole which hinders union of active ingredients utilized as amalgamation of corrosive. Maximum against virus-related, hostile to parasitic, against protozoa and hostile to malignancy sedates however are acquired from engineered sources. As a result of the re-happening resistance of pathogenic microorganisms to anti-infection agents, and also the reactions exhibited by these antimicrobials, examination of other wellsprings of antimicrobials, for example, restorative plants, for their antimicrobial properties is making progress (Abreu et al. 2012). Chemicals against heavy metals of every now and again act through various systems than ordinary anti-infection agents and could in this manner be useful in the action of safe microorganisms.

### **3.3. Plants as Medicine**

#### **3.3.1. Track of the plants having medicinal value**

Social utilization in floras for medications would be gone rear with Golden age, it is around sixty thousand years back, as indicated by residue records (Gurib-Fakim, 2006). The majority of which are still being used today to treat sicknesses running from hacks and colds to opportunistic diseases and irritation (Fabricant and Farnsworth, 2001). Medicinal services in old circumstances incorporated the utilization of leaves, blooms, stalks, fruits and underlying foundations of herbs for their helpful or restorative esteem. These medications at first took the type of rough medications, for example, solutions, teas, poultices, powders, and other natural definitions (Balunasa and Kinghorn, 2005). Learning of the particular plants to be utilized and the techniques for application for specific illnesses were approved down through oral past and data with respect to therapeutic plants was in the end logged in plants (Balick and Cox, 1996; Samuelsson 2004).

#### **3.3.2. Common uses of the traditional plants having medicinal properties**

Helpful herbs (for the most part implied as rosemary, home developed medications, pharmacological dynamic shrubs or phytomedicines) continue as mind-boggling kind of medication in numerous countries. More distant than that of the world's masses rely in a general sense of unrefined plant things to match their step by step social protection necessities (Noumi and Yomi, 2001). Most of the plants accumulated are used fresh as a piece of demand to get the focus towards the explants such as grasses, roots, blooms or regular item. On the off chance that there ought to emerge an event of woody structures, generally the wail, families and distinctive quantities are used (Barrett and Kieffer, 2001).

Some plant expels with marvelous restorative regard are the stem wool mixtures, which is used as a part of the organization of ailments, leaves of *Glyphaea brevis*, that which mollified in solvents and are utilized to resolve the intestinal ailments and hepatitis and concentrates of oil from shredding roots, rounded seeds and stalk barks of *Monodora myristica*, that treats white patch diseases, helminths, intestinal affliction and diarrhoeic issue (Gurib Fakim 2006). Various plants used for standard arrangements are right away being endorsed through consistent investigate by detachment of bioactive blends for immense use in pharmaceuticals (Samuelsson 2004). By the event, sedate exposure from remedial plants provoked in the separation of old age methodologies, for example, morphine from opium, cocaine, codeine, digitoxin and quinine, some of which are as yet

being utilized (Balunasa and Kinghorn 2005). All the more starting late a prescription,  $\beta$ -methoxypsoralen, has been conveyed from the plant *Ammi majus* (religious overseer's weeds), which was represented by Egyptian therapeutic specialists to treat vitiligo, a skin condition depicted by the loss of hues (Okpekon et al. 2004). This drug is in no time used to treat psoriasis and other skin issue, furthermore T-cell lymphoma.

### **3.4. Major metabolites of any extracts of plants**

The advantageous restorative impacts of plant resources ordinarily end from the blends in auxiliary items exhibit. Those mixes were for the most part optional metabolites, to the most part, leaves are the good stockpiling site for coveted mixes. Natural products likewise contain a generous measure of dynamic fixings, and hence are frequently expended as juice by means of oral organization to acquire the sought mixes. Different fragments of floras that could be removed from restorative mixes are branches, ethereal materials, blossoms, seeds, stem barks, and so forth. (Chan et al. 2012). Plant optional metabolites are utilized as the reason for the generation of profitable engineered mixes, for example, pharmaceuticals, beauty care products, or all the more as of late nutraceuticals. Those optional metabolites were to a great extent seen as potential wellsprings of new medications, anti-toxins, bug sprays. The results of natural noteworthiness and potential wellbeing impacts, for example, cancer prevention agent, anticancer, hostile to maturing, against atherosclerotic, antimicrobial and mitigating exercises (Bourgaud et al. 2001).

### **3.5. Mode of action of the secondary metabolites**

Plant auxiliary mixes are generally ordered by biosynthetic pathways. Three vast sub-atomic families are for the most part considered: phenolics, terpenes and steroids, and alkaloids. For a decent case in an across the board metabolite family is the phenolics, in light of the fact that these particles are included in lignin combination, these are normal for every single larger plant. Phenolic mixes are powerful cancer prevention agents also, free radicals foragers which can go about as hydrogen benefactors, decreasing operators, metal chelators and singlet oxygen quenchers. Contemplates have demonstrated that phenolic mixes, for example, catechin and quercetin are exceptionally proficient in balancing out phospholipid bilayers against peroxidation actuated by responsive oxygen species (Gülçin et al. 2010; Gülçin 2010).

It is assumed that tannins, flavonoids and other secondary metabolites are to be in charge of antidiarrheal action in expanding solvent and electrolytic reconsumption (Palombo, 2006). Terpenoids are buildup results of C5 isoprene units which are critical constituents of fundamental oils (Pichersky and Gershenzon, 2002).

It all appeared to known as dynamic invader of microscopic organisms, parasites, infections, and protozoa. The component of activity of terpenes is not completely saw but rather is theorized to include film interruption by the lipophilic mixes (Cowan 1999). The rich sources of nitrogen amounts and its metabolites are alkaloids. And are meagrely circulated into the plant majorities, yet significantly and very particular to characterized plant genera and species. This is most likely because of the restricted source of nitrogen in plants (Harborne, 1999). They are most likely because of their consequences for travel period in the small digestive system (Cowan, 1999). In research completed by Nitta et al. (2002), the dynamic concentrates got from the bark of *Shorea hemsleyana* and foundations of *Cyphostemma bainessi* were isolated into their segments and these displayed solid inhibitory movement against methicillin safe *Staphylococcus aureus*. These dynamic mixes were distinguished as stableness subsidiaries.

### **3.6. Importance of the antimicrobial testing**

In screening new antimicrobials or anti-infection agents, assessment of natural action is basic to the evaluation of weakness of pathogens to the antimicrobial specialist. Antimicrobial powerlessness testing is utilized as a part of pathological microorganisms to decide microbial resistance to certain and various antimicrobials and in pharmacognosy investigate helps to decide the viability of ancient antimicrobials as of organic concentrates beside diverse microbes (Das et al. 2010). Microorganism's development as well as its hindrance could be quantitated in various methodologies (Grare et al. 2008), e.g. practical checks, coordinate minute tallies, turbidity estimation, florescence and bio illuminator responses. From the different antimicrobial weakness strategies utilized, the circle dissemination technique and the extract's dilution strategy are normally utilized to assess the impact of the plant extricates or some other antimicrobial on ailment creating pathogens. The plate dissemination strategy is utilized as a part of deciding the zones of hindrance shown from the plant extricates, while the stock micro dilution strategy, that has been suggested by the CLSI, are utilized as a part of deciding the base inhibitory focus of plant concentrates. This strategy is fewer lumbering, a lesser amount of costly and same reproducibility when looked at the plate dispersion strategy (Grare et al. 2008). The utilization of micro titre plates permits a lot of information to be created

rapidly. Bacterial development could be surveyed either outwardly by observing the increased concentrates or by measuring optical thickness using spectrophotometer. The burden of visual appraisal of bacterial development is that it needs objectivity and exactness; though the quality of spectrometric readings might be hindered by:

- (i) added substances or antimicrobial intensifies which influence the otherworldly attributes of development media,
- (ii) the total of microscopic organisms (Eloff, 1998)., or
- (iii) Colonial colours using Colorimeter strategy, in this manner could speak to an option approach, utilizing tetrazolium salts as markers, since microscopic organisms change over them as shaded formazan subsidiaries that could be measured (Grare et al. 2008).

### **3.7. Various techniques employed for extraction**

In the examination of restorative plants, extraction is the critical initial step since it is important to extricate the wanted concoction parts from the plant materials for promote partition and optimization (Sasidharan et al. 2011). Distinctive extraction methods are accessible, yet the most well-known ones utilized as a part of plants extraction are the routine systems. In traditional extraction, the arrival of the coveted mixes generally requires absorbing and maceration mellow solvents (Chan et al. 2012). Decoction in water is comprehensively utilized in customary Chinese restorative rehearses and is a powerful strategy for consideration in situations where the nearness of a compound dissolvable is undesirable (Das et al. 2010).

Various chemicals could be utilized as a part of traditional extraction are  $\text{CH}_3\text{CO}$ , ligroin and isoheptane (Karuna et al. 2000).  $\text{LN}_2$  have been additionally utilized for some extraction and exploration work (Chen et al. 2003; Grover et al. 2000). Strategies, for example, Deep freezing and sonification are other techniques that can be utilized apart from solvent extraction. Non-ordinary techniques that can be utilized are the supercritical liquid extraction and microwave-helped systems (Chukwujekwu et al. 2009; Yang et al. 2009).

### **3.8. The techniques for forming isolates and characterization**

For the plant extricates have been gotten, recognizable proof and bioactive characterization mixes turns into a major test, on the grounds that most plant concentrates happen as a mix of different sorts of bioactive mixes or phytochemicals with distinctive



polarities (Sasidharan et al. 2011). Phytochemical screening test is a straightforward, fast, and reasonable technique that provides the scientist a fast response on the different sorts of phytochemicals or optional metabolites found in plants.

In detachment of these biologically potential mixes, distinctive chromatographic partition systems, for example, thin layer chromatography (TLC), section chromatography, streak chromatography, Sephadex chromatography and superior fluid chromatography (HPLC), might be utilized to acquire immaculate mixes (Sasidharan et al. 2011). Thin Layer Chromatography is a top pick technique for most specialists since it gives a fast reply with reference to what number of segments are there in a blend (Sumner et al. 2003). It is additionally helped to bolster the character of a metabolite in a blend and the maintenance of retention factor of a compound is looked at with the retention factor of a metabolite. The immaculate mixes were at that point utilized for the assurance of structure and natural action (Jeong et al. 2012). Various scientific strategies have been produced, which may encourage auxiliary assurance of the biologically potential compound that includes Thin Layer Chromatography, High Performance Liquid Chromatography, Liquid Chromatography / ESI pair mass spectrometry (MS/MS), slim electrophoresis, particle shower mass spectrometry, gas chromatography/MS (GC/MS), and atomic attractive reverberation. MS gives very particular concoction data that is straightforwardly identified with the concoction structure, for example, exact mass, isotope appropriation designs for basic recipe assurance and trademark part particles for basic explanation or ID through ghastrly coordinating to real compound information. In addition to the high affectability of MS permits recognition and estimation of  $10^{-12}$  to  $10^{-15}$  levels of numerous essential and optional metabolites (Lei et al. 2011).

### **3.9. Significance of this research**

The following plants are reportedly shown some antibacterial activity against the food borne pathogens. The plants chosen are *Syzygium aromaticum*, *Cannabis L.*, and *Tinospora cordifolia* (Amit pandey and Parul singh, 2011). The plants were subjected to the methanolic and ethanolic extracts and studied for the minimum inhibitory concentrations. After understanding the anti-microbial activity, the extracts were coupled with the active metal ions at different concentrations, at which shows the best inhibition against the microorganisms are confined to prepare the bio preservative.

## 4. RATIONALE AND SCOPE OF THE STUDY

### 4.1. Background of this study

Herbs and flavours have been perceived to have a wide range of dynamic constituents that display antibacterial, antifungal, antiparasitic, or potentially antiviral exercises. Fundamental oils have been utilized for quite a long time as a major aspect of regular conventional pharmaceutical. They are sweet-smelling slick fluids acquired from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, leafy foods). The real gatherings of primary segments that make fundamental oils successful antimicrobials incorporate saponins, flavonoids, carvacrol, thymol, citral, eugenol, linalool, terpenes, and their antecedents. The antimicrobial action of alliums is predominantly credited to different sorts of alk(en)yl alka/ene thiosulfonates (thiosulfonates; and their change items. Allium-inferred antimicrobial mixes restrain microorganisms by responding with the sulfhydryl (SH) gatherings of cell proteins. In olive oil, particular antimicrobial mixes including oleuropein, oleuropein aglycon, oleic corrosive and oleocanthal (not withstanding hydroxytyrosol and tyrosol) have been portrayed. The antibacterial exercises of basic oils and other plant extricates has pulled in awesome consideration for use of the unrefined extricates or their bioactive segments in nourishment biopreservation. Therefore, this may bring about nourishment corrupting and additionally antagonistic sensorial impacts to sustenance items. It has been proposed that the unfriendly sensorial impacts of basic oils operators to nourishment items can be overcome by veiling the smell with other endorsed smell mixes. Antimicrobial operators gotten from basic oils are intriguing possibility for improvement of actuated films or packaging. Plants likewise create an assortment of antimicrobial peptides, a large portion of which can be assembled in various classes: thionins, defensins, lipid exchange proteins, cyclotides. Some of them could be misused for nourishment biopreservation. Strikingly, plants can be a decent wellspring of antifungal proteins also, peptides, including chitinases, glucanases, thaumatin-like proteins, thionins,also, cyclophilin-like proteins.

So as to identify a bio preservative in the biological origin is the ultimate aim of the study. To characterize the active metabolites present in the plants and deriving the association of them with metal ions to produce an effective preservative in the biological form.

## 4.2. Statement of Problem

Preservatives are used to prevent microorganism such as, bacteria, yeast and mould growth. It is also used for preserving the colour and flavor of food product to keep food free from going bad by preventing oxidation. Avoiding preservatives is hard due to their presence in almost all food products. Food manufacturing industries used synthetic preservative almost.

Here are some of the potentially dangerous food preservatives:

### 1. Brominated Vegetable Oil Found in:

Flame Retardants and Citrus Soda Pop Food product containing: Mountain Dew, Squirt, Fanta Orange, Fresca Original Citrus, Gatorade Thirst Quencher Orange, and other soft drinks of citrus-flavored including sports drinks What it is: it is used as a flame retardant and soybeans or corn are used for their preparation by chemical companies, it is used for stabilizing the citrus oils from floating and its gives cloudy appearance to the drink.

How it can make you sick: It is band in Europe and Japan for used in food product preparation. Growth related problems are the major problem using it, while neurological and reproductive problems and skin lesions can also be seen. Normal symptoms in human beings are headaches, fatigue, memory loss, ulcers, and a loss of muscle coordination. These patients ingested much greater than average amounts of soda, but with the popularity of soft drinks like Mountain.

### 2. Sodium Nitrite and Nitrate Found in Fertilizer, Rocket Propellant and Lunch Meats Food product containing:

In meats (Processed) like bacon, hot dogs, ham, deli meats, pepperoni, salami, jerky, smoked fish and corned beef. What it is: Sodium nitrite is a mineral salt powder that prevents botulism and meats from turning grey.

How it can make you sick: on ingesting meat after cooking at high temperature lead to carcinogen forming (nitrosamines). This carcinogen can cause leukemia, colon, bladder, and pancreatic cancer. Migraine can also show as symptom. Sodium nitrates should be avoided to the pregnant woman as they can result in oxygen deprivation of fetuses.

### **3. BHA, BHT, and TBHQ Found in: Embalming Fluid and Potato Chips Food Products containing:**

Chewing gum, cereals, vegetable oil, some meats, fatty and oily foods like potato chips, butter, nuts, and snack foods What it is: Butylated hydroxyanisole and hydroxytoluene, keep oils from quickly becoming rancid. Animal feed, cosmetics, rubber products, food packaging, and petroleum products may contain this. How it can make you sick: according to Department of Health and Human Services as it is a carcinogen. Conflicting studies show the additives to either increase or decrease cancer rates in animals, but they have not been adequately reviewed and so are still —generally recognized as safe by the FDA. The creator of the Feingold diet, which eliminates most artificial food additives, claimed BHT also increased levels of hyperactivity in children.

### **4. Propyl Gallate Found in: Pesticides, Lipstick and Chewing Gum Food Products containing:**

Vegetable oil, mayonnaise, dried milk, dried meat, fresh sausage, chewing gum, chicken soup base, sports and energy drinks what it is: it is a white odorless powder. Its working is to prevent oils and fats in food products and non-edible products from spoiling. How it can make you sick: according to literature it may be work as carcinogen. The National Toxicology Program studies suggest, in rats tumors, and malignant lymphoma showed when feeding with Propyl Gallate. It should be avoided to those patients having asthma, liver or kidney problems and allergy to aspirin.

### **5. Sodium Benzoate, Benzoic Acid Find it in:**

Explosive Fireworks and Pickles Products it's in: Carbonated drinks, fruit juices and purees, salad dressings, pickles, condiments What it is: It's a salt used to preserve acidic foods. How it can make you sick: When sodium benzoate combines with vitamin C in foods, it can create benzene, a carcinogen that causes leukemia and other cancers. The risk is low, but it's there. Soft drink makers recently reformulated their fruit-flavored drinks to prevent this reaction after a lawsuit was filed against them. Those with food allergies may also experience a negative reaction, asthma or hives after ingesting, and it may increase hyperactivity in children.

## **6. Sulfites (Sodium Bisulfite, Sulfur Dioxide) Find it in:**

Volcanoes and Dried Fruit Products it's in: Wine, dried fruit, canned olives and peppers, dehydrated potatoes, cornstarch, shrimp and lobster, baked goods, condiments, molasses What it is: Sulfites inhibit microbial growth and prevent discoloration, keeping raisins brown and wine sterilized. It is also used as a bleaching agent. Sulfites destroy vitamin B1, so are prohibited in foods that are important sources of the vitamin. Sulfur dioxide is in the atmosphere as an air pollutant from volcanic gasses and coal-burning power plants. How it can make you sick: The FDA estimates that more than a million people are allergic to sulfites, chiefly asthmatics, and that they can cause mild to severe reactions such as difficulty breathing, headaches, and anaphylactic shock.

## **7. Aspartame (Also known as; E951)**

Uses: An artificial sweetener. Found in: Drinks sweeteners, Diet 'or light' anything eg. Soda and yogurts; chewing gum, cooking sauces, drinks powers, energy drinks. Can be found in certain vitamin.

Side effects/ Connected to; Headaches/ migraines, alters levels of serotonin and causes behavioral problems, Depression, Blurred vision, Eye pains, Diabetes, emotional and mood disorders; Changes the chemistry of the brain, Convulsions, Seizures, Epilepsy, Parkinson's, Alzheimer's.

Carcinogen: linked with cancers.

## **4.3. A snapshot of this research study:**

The identification of plant which potentially act as a bio preservative is identified through the series of antimicrobial studies. To proceed with, the comparative analysis to the range of microorganism that is found to be spoiling the food products are identified. The coupling of metal ions to the plant extracts are found to be characterized using chromatographic techniques, so as to identify the active metabolite behind the mode of action and the interaction it shares with the metabolite to show the effective results.

## **5. OBJECTIVES OF THE STUDY**

The main aim and objectives of my study were:

- ✓ To identify the plant metabolites.
- ✓ To form complex between plant metabolites and essential metal ions.
- ✓ To evaluate the microbicidal activity shown by the complex.
- ✓ To find the active metabolite of plant extract and the interaction it share with the essential metal ion so as to form as bio preservative.

## 6. MATERIALS AND RESEARCH METHODOLOGY

### 6.1. Equipment

The equipment's used for the present study are;

- 6.1.1. Pestle and mortar
- 6.1.2. Autoclave
- 6.1.3. Vacuum dryer
- 6.1.4. Rotary shakers
- 6.1.5. Soxhlet apparatus
- 6.1.6. Laminar air flow chamber
- 6.1.7. Bunsen burner
- 6.1.8. Weighing machine

### 6.2. Plant sample and microbial culture collection

The plant samples such as *Syzygium aromaticum* (Clove), *Cannabis* and *Tinospora cordifolia* were collected from markets of Punjab. The microbial cultures were collected from IMTECH, Chandigarh.

The Microbial Type culture collections are as follows:

- *Escherichia coli* ( Accession Number- 40)
- *Staphylococcus aureus* (Accession number- 7443)

### 6.3. Preparation of plant extracts

#### 6.3.1. Drying of plants

The plant materials collected were kept for drying to completely get rid of moisture content in it. Duration of drying performed is 4-5 days. Once the plants were found to be brittle enough to turn powder it was subjected to drying. The part of plants are:

Plant	Part used
<i>Syzygium aromaticum</i>	Expanded flowers
<i>Cannabis L</i>	Leaves
<i>Tinospora cordifolia</i>	Leaves

**Table 4. List of plant material and parts used**

### 6.3.2. Preparation of powder:

After the plants are subjected for complete drying, the plants materials were grinded using a mixer to form as a fine powder. The powder formed were again dried for 2-3 hours so as to remove other hard materials and moisture in it to avoid contamination.

### 6.3.3. Usage of solvents for extraction

The solvents used for extraction of plant materials are listed in the table as follows:

<b>Polar solvents</b>	<b>Non-Polar solvents</b>
Methanol	Acetone
Ethanol	Chloroform

**Table 5. List of solvents used for extraction**

The solvents were collected from chemical store of university departments. The methanol and ethanol were 70% and Acetone and Chloroform used are extraction type 100%.

### 6.3.4. Soxhlet apparatus

The apparatus used for extraction is Soxhlet. A Soxhlet extractor works by heating up an answer that has a solute of restricted solvency in a percolator, then cooling and gathering the condensate in a store from which the concentrated solute can be extricated. A siphon interfacing the percolator to the store flushes overabundance dissolvable once more into the supply. The solvents were used respectively for the extraction purpose. The extracts were stored in dark bottles at kept at dark place as the solvents are light sensitive.

## 6.4. Antimicrobial activity

### 6.4.1. Preparation of bacterial culture

The bacterial culture used for the study are *E. coli* and *S. aureus*. The preparation of culture media are explained as follows

#### 6.4.1.1. *E.coli* culture

Media Employed: LB broth

For setting up an immersed *E. coli* over-night culture, 5 mL LB media containing anti-biotic agents are vaccinated with a solitary state of the coveted microorganisms strain



in a 50 mL tube. Incubation is completed at 37°C with consistent shaking at 200 rpm overnight.

#### **6.4.1.2. Staphylococcus aureus culture**

Media Employed:

#### **6.4.2. Well diffusion assay**

The extracts of the plant along with the respective antibiotic and a well for distilled water were also made. The well diffusion assay explains the zone of inhibition and shown results in this experiment is furthered for the later part of the project. So, the well diffusion method plays a vital role in selection of good plant with which solvent mixed shows the high degree of resistance to the growth of food spoiling micro-organisms.

The assay was initially performed by forming four wells for a plant extract. For example, four wells were bores in the nutrient agar plates and were poured concentrations of the plant extract, antibiotic, distilled water as A, B, C named respectively to the wells bored. And after the overnight incubation, the zone of inhibition formed is measured using the regular ruler, so as to differentiate the activity of every single plant extracts could be identified and noted down.

## 7. RESULTS AND DISCUSSION

### 7.1. Plant materials for drying



**Figure 1. Drying of Cloves**

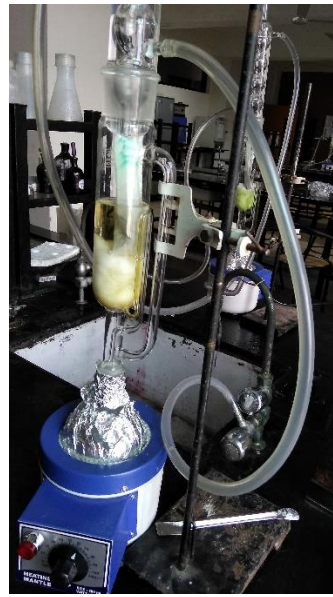


**Figure 2. Drying of *Tinospora cordifolia* leaves**



**Figure 3. Drying of *Cannabis L.* leaves**

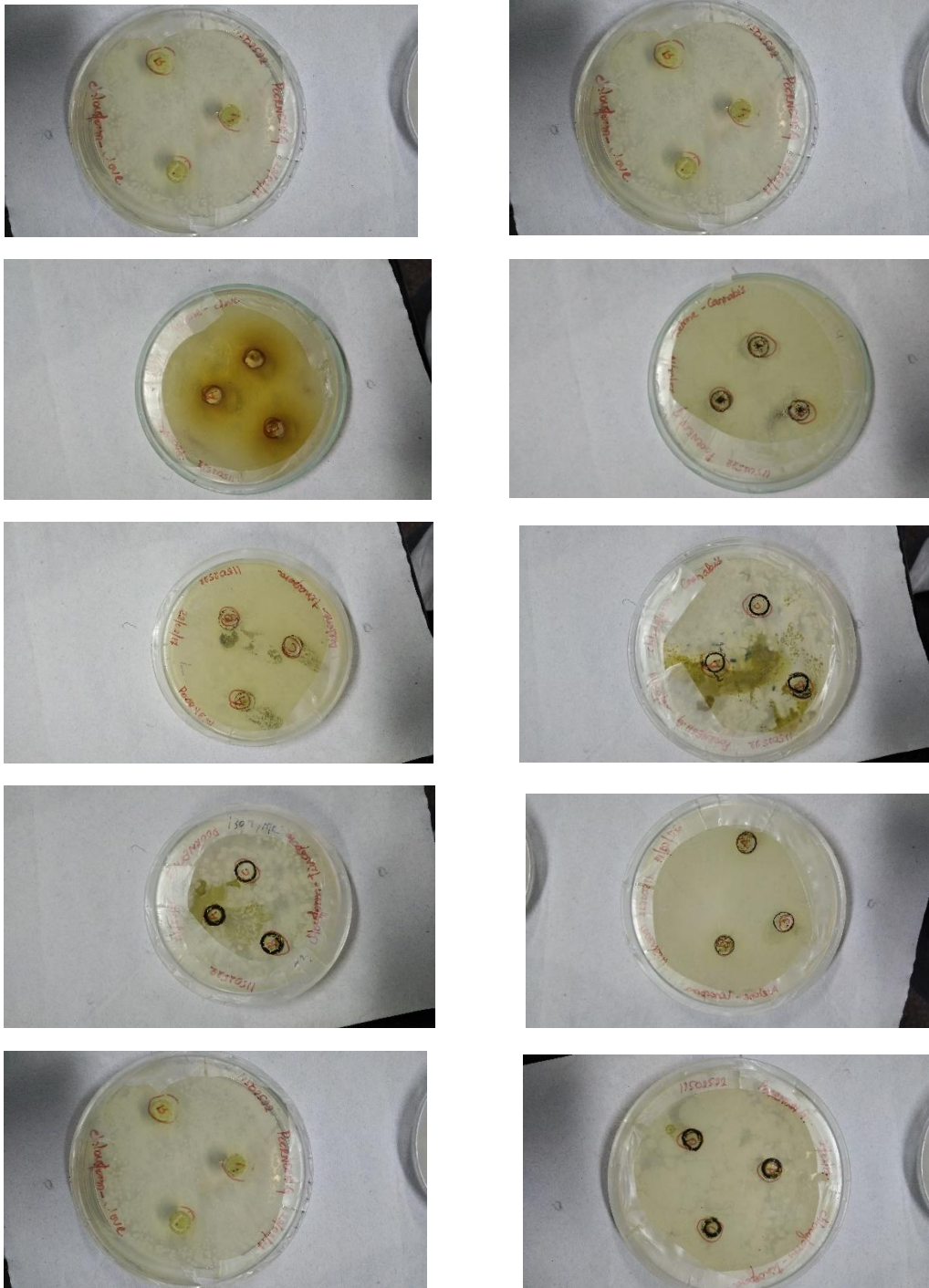
## 7.2. Soxhlet apparatus



**Figure 4. Images for the extraction experiment**

Extraction of *Tinospora cordifolia* (Top Left), Extraction of Cloves (Top Right), Extraction of *Cannabis L.* (Bottom Left) and Storage of extracts in dark place (Bottom right).

### 7.3. Antibacterial activity



**Figure 5. Antibacterial assay for the plant extracts against E.coli and S. aureus.**

(From Top Left) Clove-Acetone- E.coli, Clove-Chloroform- E.coli, Clove-Methanol-E.coli, Tinospora-Methanol-E.coli, Tinospora-Ethanol-E.coli, Cannabis-Acetone-E.coli, Cannabis-Methanol- E.coli, Cannabis- Chloroform- E.coli.

#### 7.4. Screening of bioactive compounds:

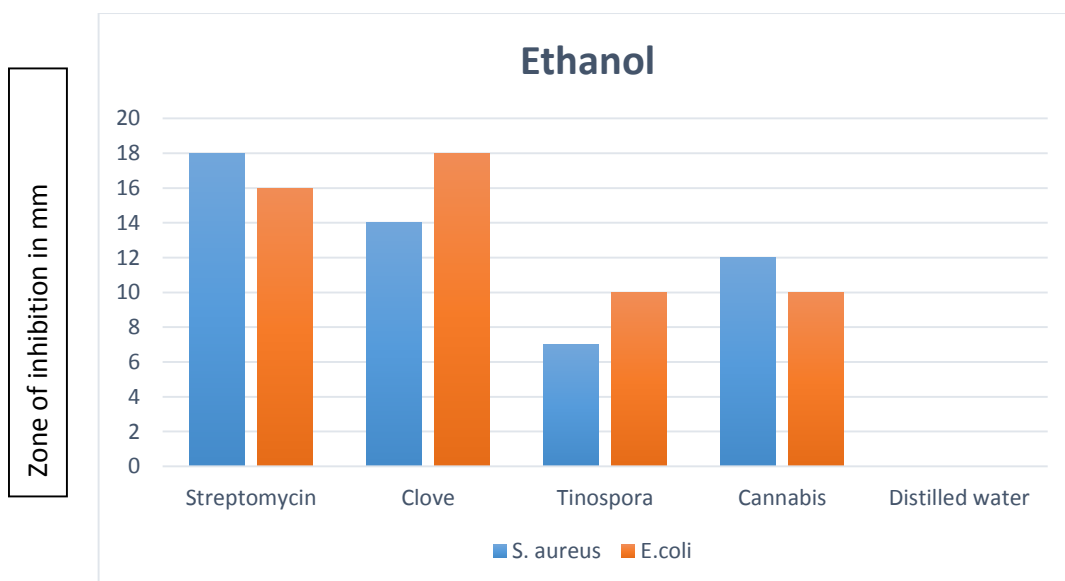
The antibacterial activity of extracts of all plants of various solvents against two food associated gram positive and gram negative bacteria was evaluated using agar well diffusion method. Nutrient agar plates were prepared for ethanolic, methanolic, acetone and chloroform solvent extracts of all the plants. 50µl of each selected bacterium was uniformly spreaded on agar plates with the help of glass spreader, after five minutes three wells approximately 5mm diameter was bored with the help of borer. The equal volume of (50µl) of antibiotic (streptomycin and amoxicillin), distilled water and plant extract were poured into the wells. The plates were incubated at 37° C for 24 hours.

#### 7.5. Zone of inhibition of Ethanolic extracts of plants

	S. aureus*	E.coli*
Streptomycin	18	16
Clove	14	18
Tinospora	7	10
Cannabis	12	10
Distilled water	0	0

\*in mm

**Table 6. Zone of inhibition of Ethanolic extract of plants**



**Graph 1. Zone of inhibition of Ethanolic extract of plants**

Zone of inhibition is measured for the ethanolic extracts of various plants against the controls streptomycin as positive control and distilled water as negative control. The values are represented in graph above.

### 7.6. Zone of inhibition of Methanolic extracts of plants

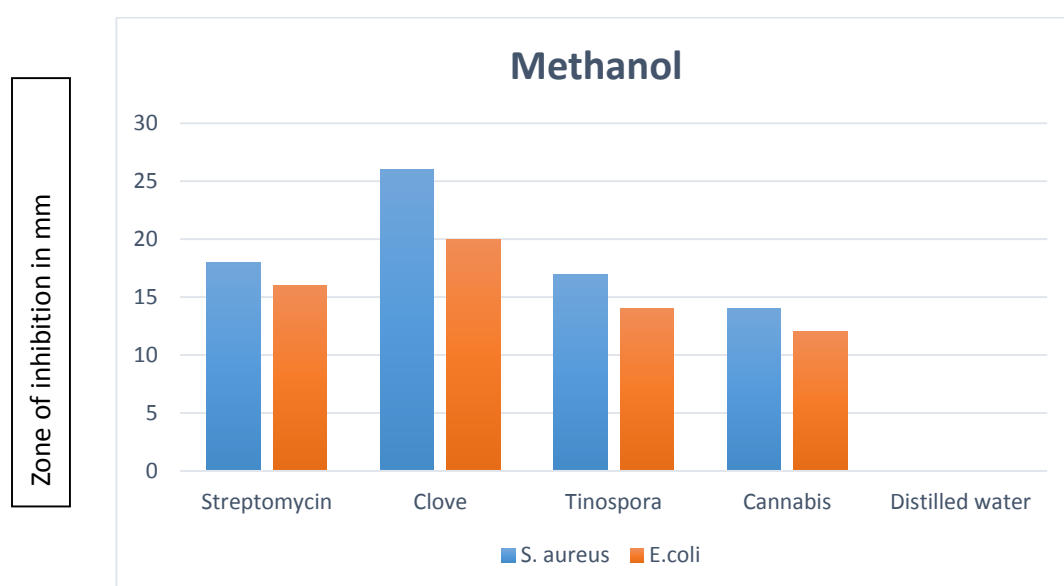
Zone of inhibition is measured for the methanolic extracts of various plants against the controls streptomycin as positive control and distilled water as negative control. The values are represented in graph below. The plant extracts were subjected to proper incubation and are poured on to the wells bored in the agar plates.

The tabulation and graphical representations are as follows.

	S. aureus*	E.coli*
Streptomycin	18	16
Clove	26	20
Tinospora	17	14
Cannabis	14	12
Distilled water	0	0

\*in mm

**Table 7. Zone of inhibition of Methanolic extracts of plants**



**Graph 2. Zone of inhibition of Methanolic extracts of plants**

### 7.7. Zone of inhibition of Acetone extracts of plants

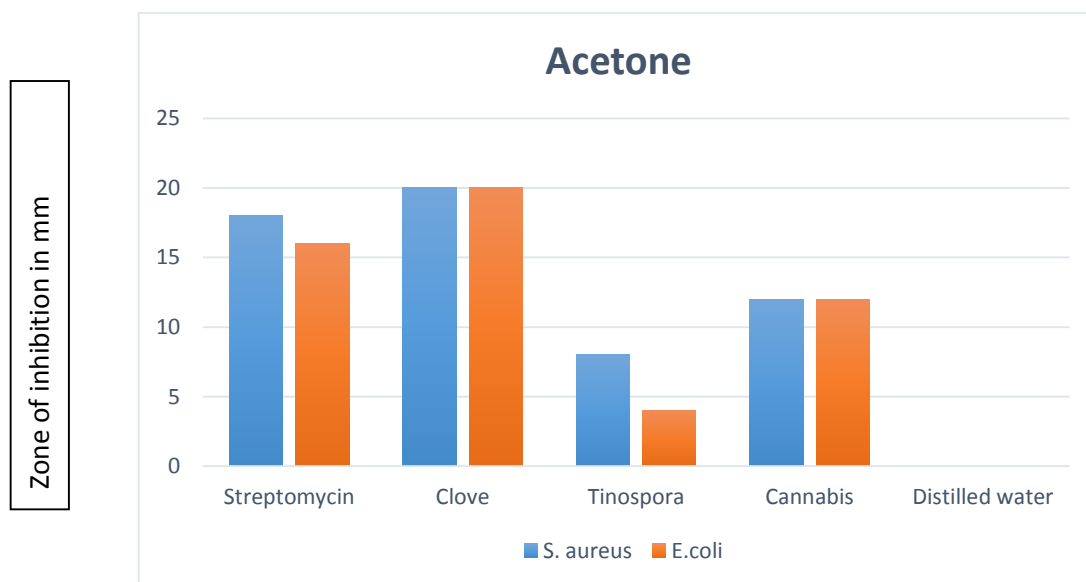
Zone of inhibition is measured for the acetone extracts of various plants against the controls streptomycin as positive control and distilled water as negative control.

	S. aureus*	E.coli*
Streptomycin	18	16
Clove	20	20
Tinospora	8	4
Cannabis	12	12
Distilled water	0	0

\*in mm

**Table 8. Zone of inhibition of Acetone extracts of plants**

The graphical representation that clearly explains the range for zone of inhibition shown by the acetone extract of plants.



**Graph 3. Zone of inhibition of Acetone extracts of plants**

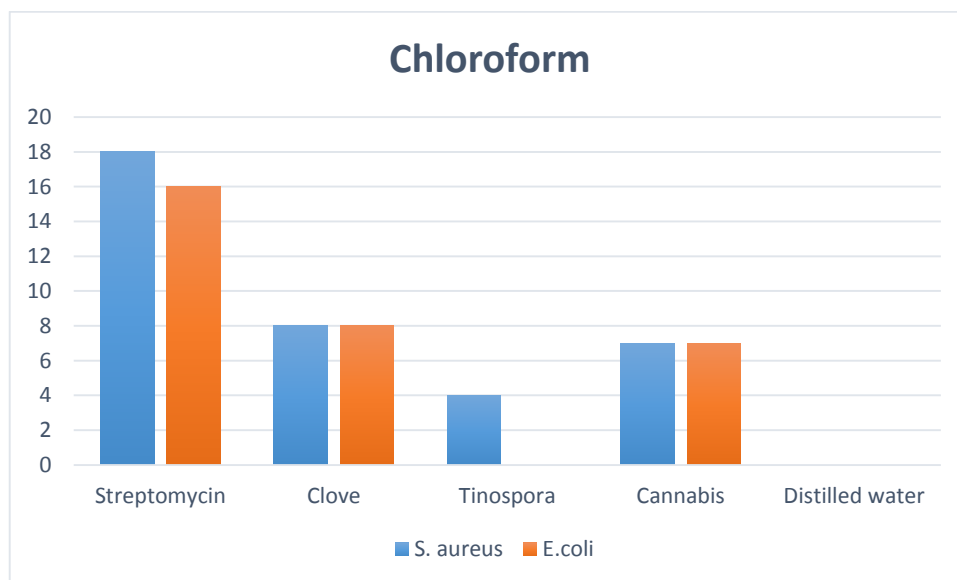
### 7.8. Zone of inhibition of Chloroform extracts of plants

Zone of inhibition is measured for the acetone extracts of various plants against the controls streptomycin as positive control and distilled water as negative control.

	S. aureus*	E.coli*
Streptomycin	18	16
Clove	8	8
Tinospora	4	0
Cannabis	7	7
Distilled water	0	0

\*in mm

**Table 8. Zone of inhibition of Chloroform extracts of plants**



**Graph 3. Zone of inhibition of Chloroform extracts of plants**

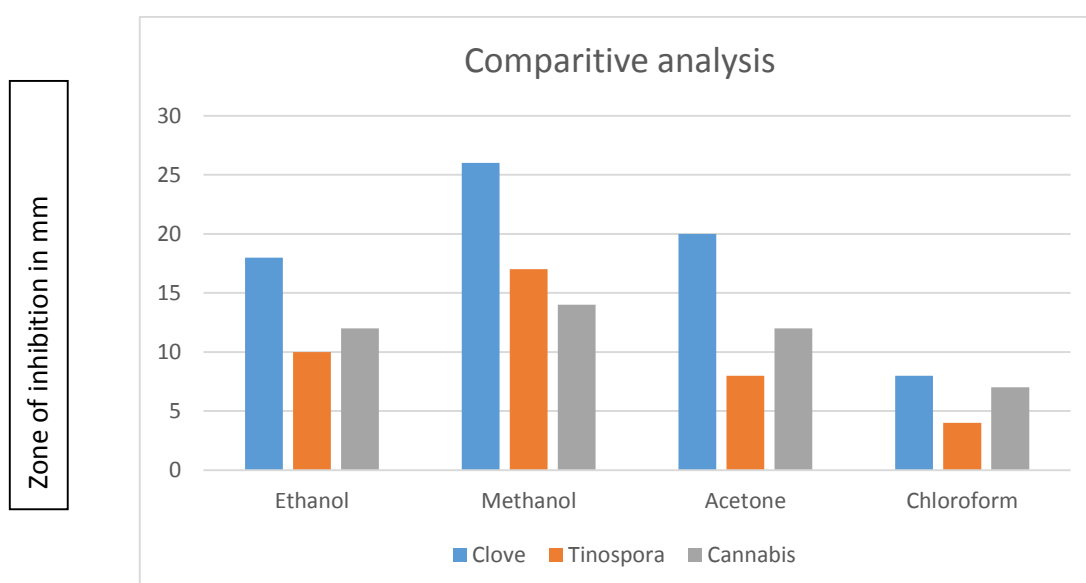


## 7.9. Comparison of all plant extracts

The most important step of this research study, in which the selection of exact plant extract that shows good zone of inhibition against the food spoiling organisms. From the table listed below, it is evident that the methanolic extract of clove plant shows the highest degree of activity than the any other plants and solvents.

	Clove	Tinospora	Cannabis
Ethanol	18	10	12
Methanol	26	17	14
Acetone	20	8	12
Chloroform	8	4	7

**Table 9. Comparison of all plant extracts**

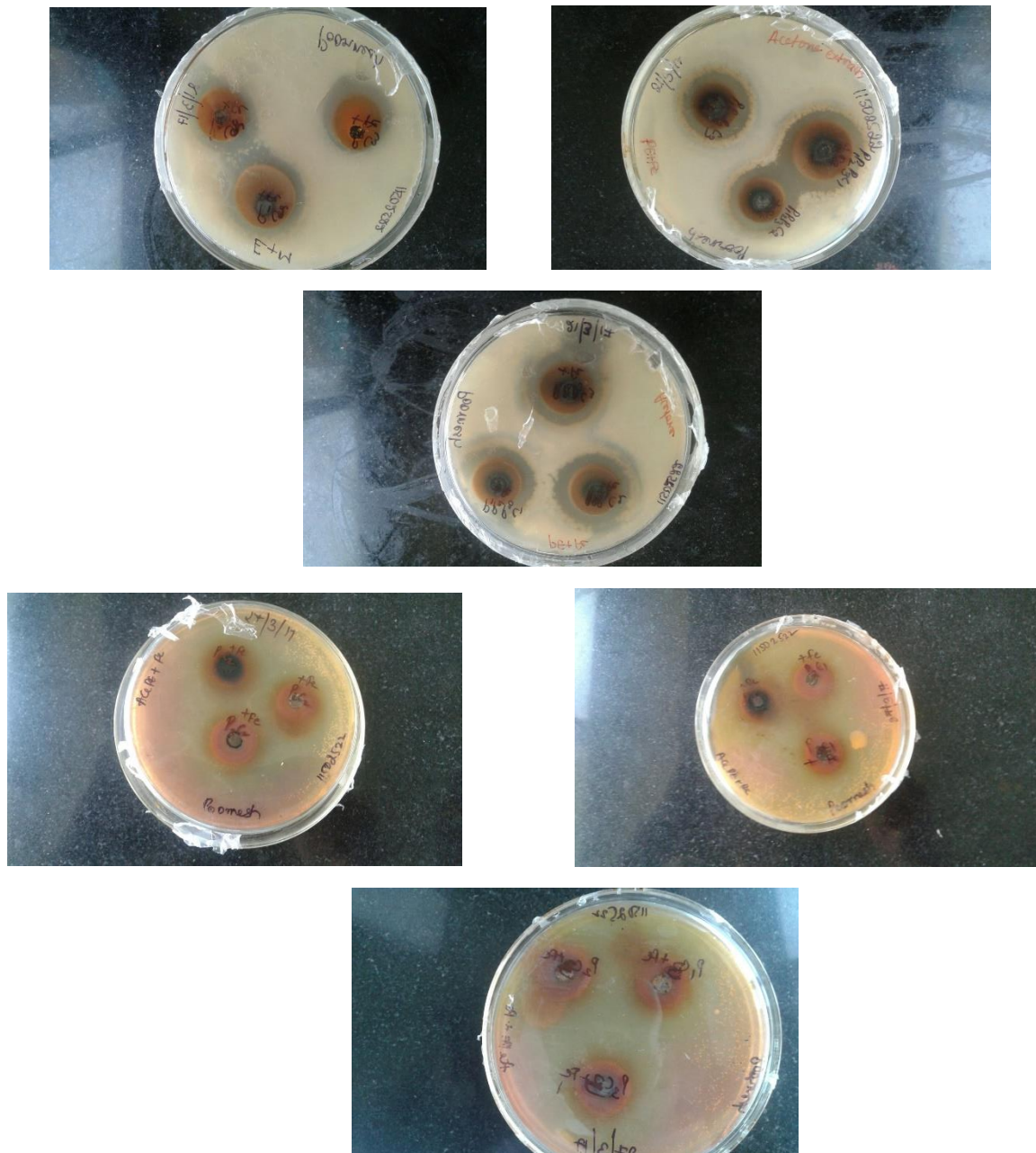


**Graph 4. Comparison of all plant extracts**

From the above analysis, it is found that the methanolic and acetone extracts of the plant clove shows better activity than the extracts of other plants. So as to produce a bio-preservative in the natural means, the methanolic and acetone extract of clove is coupled with the metal ion  $Fe^{2+}$  at various concentrations.

## 8. EXPERIMENTAL WORK

After the identification of best solvent extract that shows maximum zone of inhibition, according to the objective of this research study, I furthered the research to formulate for a bio-preservative. The proposed way is coupling of metal ion to the plant extract and again study the antibacterial activity against both the micro-organisms. The  $\text{Fe}^{2+}$  metal ions were coupled to the methanolic extract of clove and again the well diffusion method at various concentrations of  $\text{Fe}^{2+}$  + Methanolic extract of clove to the *S. aureus* and *E. coli* was performed.



**Figure 6. Antibacterial activity of Methanolic extract of clove coupled with  $\text{Fe}^{2+}$**

Plant Extract	Extract Dilution	Corresponding effect on microorganism	
		E.coli	S. aureus
Methanolic Extract of clove+ Fe <sup>2+</sup>	2g/2mL	-	-
	2g/3mL	-	-
	2g/4mL	-	-
	2g/5mL	-	-
	1 gm/2mL	-	+
	1 gm/3mL	+	+
	1gm/4mL	+	+
	1 gm/5mL	+	+
	0.5gm/2L	+	-
	0.5gm/3L	+	+
	0.5gm/4L	+	+
	0.5gm/5L	+	+

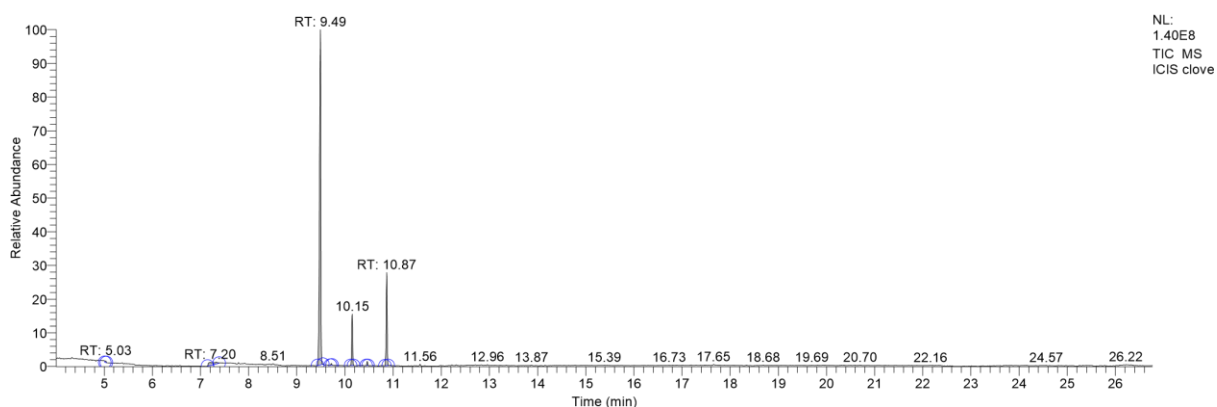
“+” refers to presence of antibacterial effect on corresponding microorganisms

“-” refers to no effect on corresponding microorganisms.

**Table 10. Effect of antibacterial activity on microorganisms for the methanolic extract of clove and coupling of Fe<sup>2+</sup> to it.**

### 8.1. Gas chromatography- Mass spectrometry

It is evident from the antibacterial activity that the methanolic extract of clove shows exemplary effect on food spoiling microorganism. To develop it as a bio preservative, the coupling of metal ion also showed the high degree of resistance against the E. coli and S. aureus. To form the biofilm, it is necessary to find the components that is responsible for the antibacterial activity and the gas chromatography helps in finding the constituents of clove extract based on the retention time and the mass spectrometry explains the concentration of the metabolites present. The samples were outsourced to study the interaction between the metal ions and clove extracts from Punjab University, Chandigarh.



**Figure 7. Gas chromatography result for clove and Fe<sup>2+</sup>**

<b>Probable Compounds</b>	<b>Retention Time</b>
C <sub>23</sub> H <sub>32</sub> O C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub> C <sub>33</sub> H <sub>47</sub> ClO <sub>7</sub>	<b>5.03</b>
C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub> C <sub>13</sub> H <sub>17</sub> N <sub>5</sub> O <sub>5</sub> C <sub>16</sub> H <sub>27</sub> NO <sub>4</sub>	<b>7.20</b>
C <sub>10</sub> H <sub>12</sub> O <sub>2</sub>	<b>9.49</b>
C <sub>15</sub> H <sub>24</sub>	<b>9.72</b>
C <sub>15</sub> H <sub>24</sub> - derivatives	<b>10.15</b>
C <sub>15</sub> H <sub>24</sub> - derivatives	<b>10.46</b>
C <sub>12</sub> H <sub>14</sub> O <sub>3</sub>	<b>10.87</b>

**Table 11. The library of compounds responsible for antibacterial activity determined by GC-MS**

## **9. CONCLUSION AND FUTURE SCOPE**

It is to conclude that the plant extract of clove shows the considerable results against the food spoiling microorganisms such as *Escherichia coli* and *Staphylococcus aureus*. According to results, it is evident that the polar solvent methanol is best suitable to prepare the plant extract and the extract shows the high zone of inhibition. Also, to produce the bio preservative in the form of biofilm any essential metal ion could be coupled to the plant extract and studied for the activity. Here, in this research the clove extracts are mixed with the  $Fe^{2+}$  metal ions and in the concentration of 0.5g/5mL and 1g/5mL shows the acceptable level of zone of inhibition.

The future work that can be carried out is to formulate the interaction shown by the library of compounds and clove extract to synthesize a biofilm that can be used for food packing and serve as a biopreservative.

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