PHYTOCHEMICAL PROFILING AND ANTIMICROBIAL POTENTIAL OF STEM AND LEAVES OF CICHORIUM INTYBUS AND MATRICARIA CHAMOMILLA

A Dissertation Report Submitted

in Partial Fulfilment of the Requirements

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MASTER OF SCIENCE

in

Biotechnology (Hons.)

By

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TOPIC APPROVAL PERFORMA

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Overall Remarks: Approved

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DECLARATION

I hereby declare that the work presented in the Thesis entitled "**Phytochemical profile and antimicrobial potential of stem and leaves of *Cichorium intybus* and *Matricaria chamomilla*"** is my own and original. The work has been carried out by me at School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India under the guidance of **Dr. Jeena Gupta (20104),** Assistant Professor (Department of Molecular Biology and Genetics) of School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India, for the award of the degree of Master of Science in Biotechnology.

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.

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CERTIFICATE

This is to certify that work embodied in this Thesis report entitled "**Phytochemical profile and antimicrobial potential of stem and leaves of *Chicorium intybus* and *Matricaria chamomilla* "** has been carried out by **Tasaduq Peerzada, Registration No.: 11402848** under my guidance and supervision**.** To the best of my knowledge, the present work is the result of his original investigation and study. No part of this thesis has ever been submitted for any other degree or diploma. The work has been carried out by him at the School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India. He has fulfilled the requirement for the award of the degree of Master of Science in Biotechnology.

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I dedicate my dissertation work to my parents and my mentor, Dr. Jeena Gupta. I sincerely appreciate her valuable time and guidance for assisting me in completing my dissertation work. She has always been the constant source of knowledge and inspiration for me and always encouraging me throughout my entire work. I'm also thankful to my institution, Lovely Professional University for providing all the facilities to accomplish my task. Last, but not the least, my parents who have always supported me and believed in me and helped me to accomplish my goals.

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ABSTRACT

Cichorium intybus (Chicory) and *Matricaria chamomilla* (Chamomile) are members of Asteraceae family and are widely distributed in Asia. They are well known traditional herbs which are included in many systems of medicine like Ayurveda, Siddha and Unani. Keeping in view their medicinal values the phytochemical, antioxidant and antimicrobial analysis of stem and leave parts were carried out. The phytochemical estimations were carried out by using standard biochemical tests. The antioxidant potential, total phenolic compounds (TPC) and total flavonoids were assessed by scavenging activity of DPPH, Folin-Ciocalteu method and aluminium chloride colorimetric assay using ascorbic acid, gallic acid and quercetin as standards respectively. The antimicrobial activity of extracts was evaluated against four pathogenic strains, namely, *Staphylococcus aureus, Escherichia coli, pseudomonas aeroginosa* and *Bacillus subtilis* by disc diffusion method. The phytochemical analysis showed the compounds like, tannins, saponins, glycosides, alkaloids, flavonoids, terpenoids etc. in aqueous extracts of both plants. Aqueous extract of Chicory stem and leaves exhibited better DPPH free radical scavenging activity with 50% inhibition at 6.01 ±0.28 µg/ml than aqueous extracts of Chamomile with 50% inhibition at 18.90±0.37 µg/ml. This corresponds to the higher flavonoid and phenol concentration in Chicory stem (17.24±0.48 µg/ml and 19.77±0.20 µg/ml respectively) and leaves (18.36 ±0.32µg/ml and 10.8±0.12µg/ml respectively) than *Matricaria chamomilla (*11.5±0.56µg/ml total flavonoid concentration and 19.28±0.87µg/ml total phenol concentration in leaves and 7.76±0.32µg/ml and 10.47±0.46µg/ml in stem respectively). Further maximum antimicrobial activity was seen in leaves and stem extracts of *Cichorium intybus* against all four strains with zones of inhibition measuring 25.5±0.28 mm (*E.coli*),25.8±0.08 mm (*P.aeruginosa*),28.7±0.14 mm (*S.aureus*),26.2±01 mm (*B.subtilis*)in leave extracts and 23.9±0.05 mm (*E.coli*),22.6±0.3 mm *(P.aeruginosa*),24.6±0.3 mm (*S.aureus*), 24.7±0.1mm (*B.subtilis*). The zones of inhibition of leaf and stem extracts of *Matricaria chamomilla* were found to be 23.7±0.40 mm (*E.coli*), 24.9±0.05 mm *(P.aeruginosa*), 21.8±0.1 mm (*S.aureus*), 23.9±0.05 mm (*B.subtilis*), and 9.9±0.5 mm (*E.coli*), 27.4±0.3 mm (*P.aeruginosa*), 22.7±0.4 mm (*S.aureus*), 9.2±0.1 mm *(B.subtilis*) respectively. Therefore, it can be concluded from the results that *Cichorium intybus* and *Matricaria chamomilla* consist of natural antioxidants and antimicrobials and thus have the potential to be used for the treatment of various diseases.

ABBREVIATIONS

DPPH	1,1- Diphenyl-2-picryl-hydrazyl
$AlCl_3$	Aluminium chloride
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
KCH_3COO	Potassium acetate
Na_2CO_3	Sodium carbonate
KOH	Potassium hydroxide
COOH	Carboxylic acid
$FeCl_3$	Ferric chloride
FCR	Folin-Ciocalteu reagent
UV	Ultraviolet
LAF	Laminar air flow
NA	Nutrient agar
l	Liters
mg	Miligrams
g	Grams
µl	Microliters
%	Percentage
nm	Nanometers
mm	Milimeters
ZOI	Zone of inhibition
˚C	Degree centigrade
Temp.	Temperature

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CHAPTER 1

INTRODUCTION AND PROBLEM BACKGROUND

Phytochemicals are diverse group of molecules widely distributed in plants. These chemicals have gained attention since ancient times owing to their multitude effects on human health, physiology and economic importance. These chemicals include saponins, tannins, flavonoids, glycosides, terpenes, etc. whose exact biosynthesis and functions are still largely unknown. Most of these secondary metabolites can be broadly classified into three main classes: Alkaloids, Phenolics, and Terpenoids. These diverse groups possess several modifications resulting in large number of secondary metabolites. Considering this, it is thus no surprise that large attention is focused on identification of plants with putative rich phytochemicals and their characterization for medicinal and economic value. In this regard,

Cichorium intybus and *Matricaria chamomilla* are of great importance due to documented presence of several phytochemicals in these plants.

Cichorium intybus (Chicory) and *Matricaria chamomilla* are members of Asteraceae family which are widely distributed in Asia. These plants have been implicated for their medicinal importance owing to presence of diverse variety of secondary metabolites which include flavonoids, alkaloids, terpenes, saponins, and tannins (Molan, et al. 2003). Several different Chicory species have gained importance in agriculture due to their highest market value and potential to treat many diseases (Poli, et al. 2002). In many countries during harsh winter times when fresh vegetables are not available, Chicory can be consumed in raw salads and included in daily meal. Chicory has a great potential to survive low temperatures and therefore offers a great resistance to decaying processes (Rosetto, et al. 2005). The leaves of Chicory consists sufficient amounts of phenols, vitamins, calcium, phosphorus, and potassium (Mulabagal, et al. 2009). In addition, Chicory consists of cichoric acid that helps to

activate our immune system and therefore preventing allergic reactions, inflammation and bacterial infections to certain extent (Nayeemunssia, et al. 2009). In ancient times, *C. intybus* has been extensively used for the treatment of headache, diarrhoea, gallstones, and jaundice (Afzal, et al. 2009). Also other reports have confirmed that *C. intybus* has antibacterial (Nandagopal and Rajitha Kumari, 2007), anti-inflammatory (Cavin, et al. 2005), anti-ulcerogenic and hyperglycaemic activities (Rifat-uz-Zaman et al. 2006). The antioxidants present in the Chicory may prevent oxidative damage to macromolecules in the human system including proteins, nucleic acids, and lipids by neutralizing several different free radicals which are produced as a part of various metabolic reactions (Shui and Leong, 2004). The free radicals are generated during metabolic pathway reactions have potential to damage macromolecules and cause activation of cell death mechanism which can lead to various physiological, cardiovascular and other physiological disorders (Uttara et al. 2009). The various classes of secondary metabolites which include phenolic acids, flavonoids, ascorbic acid and tocopherols and other phytochemical compounds possess many pharmacological properties, for example, antioxidant potential, anti-cancer, anti-diabetic and anti-inflammatory activities and therefore have been used for the treatment of various ailments (Bergman et al. 2001).

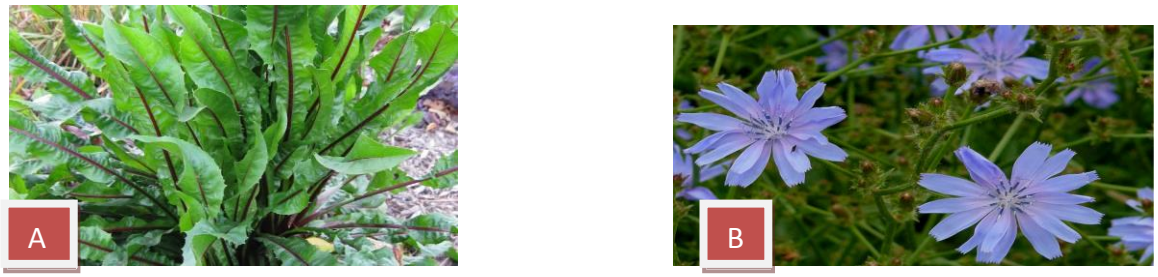

Figure 1 : **A-** *Cichorium intybus* plant **B-** *Cichorium intybus* flowers

Matricaria chamomilla, also known as Chamomile is an ancient medicinal herb belonging to Asteraceae family. Similar to Chicory, Chamomile is considered of medicinal value and has been implicated for many pharmacological properties which include antiseptic, anti-inflammatory, antispasmodic and mildly sudorific (Mericili, et al. 1990). A special product of *Matricaria chamomilla* known as Chamomile oil is widely used and it has a high market value in Europe due to its antibacterial and antifungal properties and is also used as sedative agent and in indigestion (Gould L, Reddy CV, 1973).

Despite these observations, our knowledge pertaining to phytochemicals profile in different parts of these plants is limited. There is lack of comparative qualitative and quantitative data regarding various phytochemicals in *Cichorium intybus* and *Matricaria chamomilla.* This project thus aims at identification of putative phytochemicals from stem and leaves of *Cichorium intybus* and *Matricaria chamomilla* followed by quantification and separation of polyphenols in these plants. This will be followed by analysis of anti-microbial activities in plant extracts.

Figure 2 : **A**- *Matricaria chamomilla* plant **B**- *Matricaria chamomilla* seeds

CHAPTER 2

SCOPE OF THE STUDY

This study involves the phytochemical profiling of plant extracts of *Chicory intybus* and *Matricaria chamomilla* followed by total polyphenol contents, total flavonoids, and evaluation of their antimicrobial, antifungal, and antioxidant properties. It will allow us to determine their different pharmaceutical properties and therefore have the potential to be used in development of alternative medicines for the treatment of various diseases.

CHAPTER 3

PROPOSED RESEATCH OBJECTIVES

Keeping in view the foregoing discussion, the present project has been formulated with the following objectives:

1. Analysis of phytochemical profile in stem and leaves of *Cichorium intybus* and *Matricaria chamomilla*.
2. Extraction and characterization of total polyphenols in aqueous plant part extracts.
3. Evaluation of anti-oxidative potential of stem and leaves of *Cichorium intybus* and *Matricaria chamomilla*.
4. Analysis of antimicrobial potential of plant polyphenolic extracts.

CHAPTER 4

REVIEW OF LITERATURE

4.1 Introduction:

The phytochemicals or secondary metabolites are natural biological compounds which are produced by diverse plant species which do not have direct role in the growth and development of plants, however their absence may lead to different kinds of diseases in the plants. They have a role in defence mechanisms of the plants and have diverse biological functions and chemical structures. In addition, these phytochemicals increase the medicinal importance of the plants and are therefore responsible for providing defence system to the plants. These metabolites have been reported to have action on human systems.

Plants produce about 100,000 different secondary metabolites of globally known 400,000 to 500,000 plant species. They include structures of more than 15000 alkaloids, 30,000 terpenes, several thousand phenyl propanoids, 1000 flavoniods, 500 quinones, 700 polyacetylenes, and 800 non-protein amino acids which have been characterized (Cowan, 1999).

Cichorium intybus (Chicory) and *Matricaria chamomilla* are members of Asteraceae family which are widely distributed in Asia. These plants have been implicated for their medicinal importance owing to presence of diverse variety of secondary metabolites among which flavonoids, alkaloids terpenes, saponins, and tannins have gained much of the importance (Molan, et al. 2003). Several different species of Chicory are important and grown in agricultural crops due to their high market value and yield (Poli, et al. 2002). Chicory has the great potential to survive low temperatures and therefore is considered to be a great vegetable during harsh winter when many vegetable crops cannot grow. It can be eaten raw in salads and included in daily diet due to its high nutritious value (Rosetto et

al. 2005). The Chicory leaves consists sufficient amounts of phenols, vitamins, calcium, phosphorus, and potassium (Mulabagal, et al. 2009). In addition, Chicory contains an important compound called cichoric acid having the ability to activate the immune system and therefore can prevent many allergic reactions, inflammation and bacterial infections to certain extent (Nayeemunssia, et 2009). In ancient times, *C. intybus* has been extensively used for the treatment of headache, diarrhoea, gallstones, jaundice and many other ailments (Afzal, et al. 2009). The other reports have shown that *C. intybus* has antibacterial (Nandagopal and Rajitha Kumari, 2007), anti-inflammatory (Cavin, et al. 2005), anti-ulcerogenic and hyperglycaemic activities (Rifat-uz-Zaman et al. 2006). The antioxidants present in the Chicory protects macromolecules such as proteins, and nucleic acids and lipids from oxidative damage which is generally caused to the generation of free radicals in metabolic reactions. These antioxidants have the ability to neutralize the free radicals and prevent cell damage and cell stress (Shui and Leong, 2004). The free radicals can otherwise react with lipids, proteins, and nucleic acids and cause activation of apoptotic pathway signalling which ultimately leads to various neurological, cardiovascular and other physiological disorders (Uttara et al. 2009). The most important subclass of secondary metabolites including phenolic acids, flavonoids, ascorbic acid and tocopherols have been well-known to many pharmacological properties such as antioxidant potential, anti-cancer and anti-diabetic properties and are used for the treatment of various ailments (Bergman et al. 2001).

Matricaria chamomilla, also known as Chamomile is an ancient medicinal herb which belongs to Asteraceae family, most frequently known as "star plant among medicinal species". Similar to Chicory, Chamomile consists of multiple pharmacological properties with most discussed being an anti-inflammatory, antiseptic, antispasmodic and mildly sudorific (Mericili, et al. 1990). A very special product of *Matricaria chamomilla* known as

chamomile oil is widely being used in European countries due to its amazing antibacterial and antifungal properties and is also used as mild sedative and in indigestion problems (Gould L, Reddy CV, 1973).

The plants secondary metabolites are generally categorized into three main classes:

1.Phenolics.

2.Terpenoids

3.Alkaloids

4.2. Phenolics

They are classified on the basis of presence of at least one aromatic ring in their chemical structure with one or more hydroxyl group.

4.2.1. Biosynthesis

Most of the phenolics that are known till date have been isolated from plants. They are generally biosynthesized through the shikimate pathway. The shikimate pathway requires precursors like phosphoenolpyruvate (PEP) and erythrose-4-P derived from glycolysis and pentose phosphate pathway respectively (Cowan, 1999). These precursors are essential and are involved in the biosynthesis of phenolic compounds in the plants. The mechanism involves the condensation of these two precursors or compounds to form a six-carbon cyclic compound with one carbon with COOH group side chain called shikimate. This shikimate is then phosphorylated with another molecule of PEP to produce a cyclic compound containing three carbon and one carbon side chains. This is finally converted to aromatic amino acids, that is, phenylalanine and tyrosine. These amino acids are then deaminated followed by hydroxylation at different carbon atoms in the aromatic ring to form cinnamic acid derivatives which are utilized to form different phenolic compounds.

4.2.2. Important known functions of Phenolics

- Phenolics are the essential as cell wall components. They form cell wall structures like cutins, suberins, and lignins which provide mechanical support and act as barriers against microbial infections.
- Phenolics have defensive role in plants by providing protection against predators. Phenolics may accumulate as low molecular compounds called as phytoalexins due to microbial attack.
- The flavonoids and anthocyanins are responsible for the flower and fruit colour.
- Among these phenolic phytoalexins, hydroxycoumarins and hydroxycinnamate have contribution in disease resistance in plants.
- Phenolics also act as signalling molecules in the interaction of nitrogen-fixing bacteria and leguminous plants.

4.3. Terpenoids

Secondary metabolites called terpenoids are the most diverse and the largest family of organic compounds with over 23,000 known structures. Their structures range from simple structures to complex polycyclic compounds with size variation from five-carbon hemiterpenes to natural rubber, consisting of thousands of units of terpenes.

4.3.1 Biosynthesis

They are derived from polymeric isoprene units and synthesized by mevalonic acid pathway. Their synthesis generally occurs through the fusion of small isoprene units, and are classified on the basis of number of five-carbon units consisting their full structure (Mahmoud et al. 2002). Several of aromatic and flavour compounds, such as linalool, methanol, geraniol and caryophyllene are derived from monoterpenes (C10) with just couple of isoprene units, and sesquiterpenes (C15), with three isoprene units. Few of compounds like steroids, carotenoids, and gibberlic acid are just some of its members.

4.3.2 Important Biological Functions of Terpenes

- They possess the antimicrobial and insecticidal properties and have been utilized as pesticides and fungicides in agriculture and horticulture.
- A number of diterpenoids are well known for their biological/ pharmacological/ therapeutic effects.
- Some of the bioactive diterpenes include ginkgolides, gibberellins, phorbol esters, and an anticancer agent, paclitaxel.
- Biologically, monoterpenes possess a variety of biological effects including antibacterial, sedative, anti-tumor, cytotoxic, anti-inflammatory, insecticidal, and others.

4.4. Alkaloids

They are a very huge group of plant secondary metabolites with more than 12,000 compounds isolated so far. They generally contain nitrogen atoms in their structures. In addition to the elements like carbon, hydrogen, and nitrogen, they may also contain oxygen, sulphur, bromine, chlorine and phosphorous rarely. Alkaloids have been classified due to their immense structural diversity and there is no proper classification of them.

4.4.1. Biosynthesis:

The amino acid, tyrosine is generally involved in the biosynthesis of alkaloids. One of the important examples include the biosynthesis of morphine which includes a phenol coupling reaction involving a benzylisoquinoline alkaloid.

4.4.2 Biological functions of alkaloids

- They possess defence mechanisms against predators, especially mammals.
- An alkaloid, tropane has been shown to prevent intestinal spasms and act as stimulant of central nervous system.
- Isoquinoline has been found to be analgesic and used in the treatment of coughs.
- Catharanthus vinblastine and vincristine are commonly used for cancer therapy by binding to microtubules and inhibit hydrolysis of GTP and thus arresting cell division at metaphase.
- Vinblastine is a component of chemotherapy for metastatic testicular cancer, Hodgkins disease, and other lymphomas.
- Vincristine is the preferred treatment for acute leukemia in children.

4.5. Key Derivatives of Secondary Metabolites

4.5.1. Flavonoids:

Flavonoids are amongst the principal classes of polyphenols generally present in vacuoles of plant cells. They have been categorized in three main gropus : anthocyanins, flavones, and flavonols. They are ubiqutous in plants and fulfil multiple functions, like flower colouration, producing yellow, red or blue pigmentation in petals designed to attract pollinator animals. The other important functions of flavonoids include ultraviolet filtration, nitrogen-fixation and floral pigmentation in higher plant species. They can also function as hormones, physiological regulators and cell cycle inhibitors.

4.5.2. Tannins:

Tannins are polyphenolic organic structures that are involved in precipitation of proteins. They are made up of different types of oligomers and polymers. They can from complexes with proteins, cellulose, starch and minerals. Their synthesis occurs via a

pathway called shikimic acid pathway. Except few of tannins with higher molecular weight, all other compounds can solubilise in water. They have been categorized into two subgroups: hydrolysable tannins, which include gallotannins, elligatannins, condensed tannins and complex tannins.

4.5.3. Glycosides:

They may be alcoholic, phenolic or sulphur containing compounds. They have been classified on the basis of presence of sugar moiety linked to a special bond to one or non-sugar portions. Several plants store chemicals in the form of inactive glycosides, which can be stimulated by enzyme hydrolysis. Due to this reason most glycosides can be categorized as pro-drugs since they remain inactive until they are hydrolyzed.

4.5.4 Saponins:

Saponins are biological molecules that make colloidal solutions in water and produce lather on shaking and precipitate cholesterol. They are found as glycosides whose aglycone is tripenoid or steroidal structure.

CHAPTER 5

PROPOSED RESEARCH METHODOLOGY

5.1. Equipments Required :

Electric grinder

Hot air oven

Digital weighing balance

Laminar air flow

Micropipette

Heating mantle

Spectrophotometer

Water bath

Incubator bacteriological

Shaker Incubator

pH meter

5.2. Materials Required :

Plant samples

Conical flasks

Eppendorf tubes

Falcon tubes

Glass beakers

Test tubes

Membrane filters

Microtips

Petri plates

Well borer

Whatman paper

5.3. Chemicals Required :

Mayer’s reagent

Hydrochloric acid

Molisch’s reagent

Sulphuric acid

Sodium hydroxide

Ferric chloride

Potassium hydroxide

Ethanol absolute

Acetic anhydride

Chloroform

Aluminium chloride

Potassium acetate

Quercitin

Folin-Ciocalteu Reagent (FCR)

Sodium carbonate

0.175 mM DPPH solution

Ascorbic acid

Nutrient Agar

5.4. METHODOLOGY

- Stem and leaves of *Cichorium intybus* and *Matricaria chamomilla* were procured from my native place in Srinagar. The aqueous plant extracts were used to assess profile of following phytochemicals using standard biochemical tests:

  (i) Saponins

  (ii) Glycosides

  (iii) Alkaloids

  (iv) Flavonoids

  (v) Phenols

  (vi) Phlobatannins

  (vii) Reducing sugars

  (viii) Steroids

  (ix) Tannins

  (x) Terpenoids

- Total polyphenols and flavonoids in stem and leaves of various plants were assessed by spectrophotometric methods.
- Polyphenolic compounds and their derivatives were also analysed for their potential antibacterial activity by using standard microbiological methods.

- The research should provide new information regarding the variations and diversity of phytochemicals in different plant parts of *Cichorium intybus* and *Matricaria chamomilla.*
- The research will help identify plant parts with high polyphenolic content that can be used as putative antimicrobial or antioxidant agents.

5.4.1. Collection of Plant Material:

The plants, *Chicory intybus* and *Matricaria chamomilla* were collected from my native place, Srinagar, Kashmir in September, 2015. The plants were immediately stored in the refrigerator after their collection. The plant samples were thoroughly washed with water and then rinsed with distilled water. After this, the plants were dried in the hot air oven for 2-3 hours at 40˚C. The dry weight of stem and leaves of each plant weighed 250 grams. The leave and the stem parts of both the plants were separated and subsequently crushed in the mortar and pestle to a fine powder and stored in sealed polythene bags in refrigerator for further use.

5.4.2. Preparation of Aqueous extracts of stem and leaves:

The aqueous extracts of stem and leave of the plants were prepared in 500 ml conical flasks by initially dissolving 20 gram powder of stem and leave of both plants in 100 ml of distilled water and mixed thoroughly. However, this wasn't sufficient for the extraction and therefore more water was added until the contents mixed properly. The final volume made is 250 ml. All of these mixtures were kept in an orbital shaker initially for 24 hours at 28˚C with 60 rpm. This didn't give the sufficient extraction and subsequently the incubation time was extended to 72 hours. It was followed by filtering all of these extracts through two-folds of muslin cloth and the filtrates were collected in 250 ml conical flasks. The stem and leave extract of Chicory intybus was found to be 155 ml and 95 ml respectively. Similarly, that of *Matricaria chamomilla* stem and leave extracts were found to be 175 ml and 185 ml respectively. All of these filtrates were stored in the refrigerator at 4˚C for further use.

5.4.3. Phytochemical Analysis:

The stem and leave extracts of both the plants were studied for various phytochemicals. The symbols like ± , +, ++ and +++ determine present in traces, present, moderately present and present in abundance respectively.

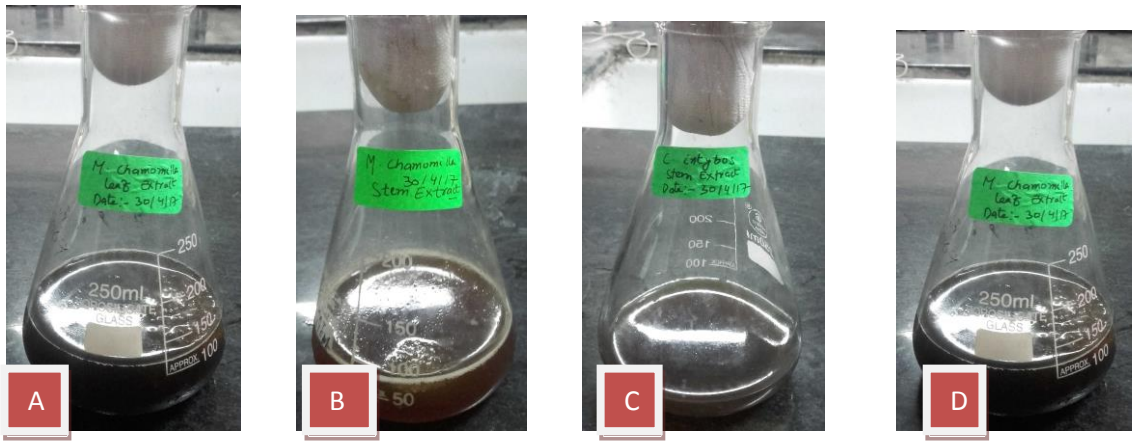

Figure 3 : Aqueous extraction carried out in shaker incubator at 25˚C for incubation time of 72 hours. **A**- *Matricaria* leaf extract; **B**- *Matricaria* stem extract; **C**- *Chicory* stem extract; **D**- *Chicory* leaf extract.

Test for alkaloids :

Mayer's reagent test : To the 3 ml of extract 1 ml of 1% HCl was added in a test tube. The mixture was slowly heated for 20 minutes in a water bath, allowed to cool and filtered. After this, two drops of Mayer's reagent was mixed in 1 ml of filtrate and observed for turbidity or creamy precipitates.

Test for carbohydrates :

Molisch test : 2-3 drops of Molisch's reagent (10% of 1-napthol in ethanol) were added to the 1 ml of aqueous extract. The test tube was slightly bent over and 1-2 ml of conc. H_2SO_4 was added carefully along the sides of a test tube and observed for the formation of red colored violet ring at the junction.

Test for flavonoids:

NaOH test: 3 ml of the extract was treated with 1 ml of 10% aqueous NaOH solution. A strong yellow color was formed which turned colourless on the addition of dilute HCl, confirming the presence of flavonoid compounds.

H_2SO_4 test: A few drops of the conc. H_2SO_4 were added to 1 ml of extract along the edges of the test tube. The formation of yellow colour indicated the presence of flavonoids.

Test for glycosides:

$FeCl_3$ test: 2.5 ml of extract was allowed to react react with 5 ml conc. H_2SO_4 and boiled for 15 min in water bath. The above mixture was cooled and neutralized with 20% KOH. Three drops of $FeCl_3$ was added to one half of the mixture and observed for precipitation of green or black color.

Test for gums and mucilage :

1 ml of extract was mixed with 2.5 ml of absolute alcohol under continuous stirring leading to the formation of precipitates confirming the presence of gums and mucilage.

Test for phenol:

$FeCl_3$ test : 2-3 ml of extract was treated with few drops of 10% aqueous $FeCl_3$ and observed for emergence of blue green colour.

Test for phlobatannins:

A few drops of 2% aqueous HCl were added to 1 ml of extract and boiled for few minutes. The formation of red precipitates determines the presence of phlobatannins.

Test for reducing sugars :

Fehling solution A and B 2-3 ml each were heated gently and allowed to cool. It was followed by addition of 1 ml extract. The mixture was boiled for 5-10 min. Formation of brown red precipitates detected the presence of reducing sugars.

Test for saponins:

Froth test : In a test tube 2 ml of extract was taken and shaken vigorously and observed for formation of froth.

Test for steroids :

Liebermann-Burchard test : 1 ml of extract and 1 ml of chloroform were mixed together. About 2-3 ml of acetic anhydride was added to the mixture. Two drops of conc. H_2SO_4 was added along the edges of the test tube and observed for blue green or dark green colour.

H_2SO_4 test : 6-7 drops of conc. H_2SO_4 was added to 1 ml of extract from the edges of the test tube. The development of red colour indicated the presence of steroids.

Test for tannins :

$FeCl_3$ test : 1 ml of extract was reacted with 1-2 drops of $FeCl_3$ and observed for dark green colour.

KOH test : 1 ml of freshly prepared 10% KOH was added to 1 ml of extract. Dirty white precipitates indicated the presence of tannins.

Test for terpenoids : Salkowaski test : 1 ml of extract was reacted with 2 ml of chloroform. About 3 ml of conc. H_2SO_4 was added carefully from the edges of the test tube. Formation of reddish brown color at interface confirmed the presence of terpenoids.

5.5. DPPH Free Radical Scavenging Activity :

The free radical scavenging activity of the aqueous extracts of stem and leaves of both plants was measured by 2,2'- diphenyl-1-picrylhydrazyl (DPPH) according to the method described by (Sahare P. et al, 2014). The scavenging activity was assessed based on percentage of DPPH radical scavenged by following equation :

$$\text{Scavenging effect (\%)} = \frac{A_{Control} - A_{Extract}}{A_{Control}} \times 100$$

5.5.1. Preparation of Standard :

0.05 mg/ml stock solution of ascorbic acid was prepared by dissolving 5 mg of ascorbic acid powder in 100 ml of distilled water. This standard solution was taken in 16 different test tubes in eight different concentrations (20 µl- 160 µl/ 1 mg-8 mg) in duplicates as shown below in the *table 1*. To each tube 1 ml of freshly prepared 0.l75 mM of DPPH solution (prepared by dissolving 4 mg of DPPH powder in 75 ml of methanol) was added. The solutions were mixed thoroughly, covered with silver foil and kept in dark for half an hour. The absorbance was recorded at 517 nm using UV-Visible spectrophotometer. Methanol was used as a blank.

5.5.2. Preparation of Extract Dilutions :

The extract dilution was carried out by taking eight different concentrations of plant samples (20 µl- 160 µl) and dissolved in distilled water to make up final volume of 1 ml. Each of the diluted sample was taken in a duplicate. To each test tube, 1 ml of freshly prepared 0.175 mM of DPPH solution was added. All of the tubes were thoroughly mixed and wrapped with silver foil and kept in dark for half an hour. The absorbance of the samples was taken at 517 nm using UV-Visible spectrophotometer.

S. No.	Ascorbic Acid	Distilled Water	DPPH Solution
S1	20 µl	1980 µl	1000 µl
S2	40 µl	1960 µl	1000 µl
S3	60 µl	1940 µl	1000 µl
S4	80 µl	1920 µl	1000 µl
S5	100 µl	1900 µl	1000 µl
S6	120 µl	1880 µl	1000 µl
S7	140 µl	1860 µl	1000 µl
S8	160 µl	1840 µl	1000 µl

Table 1 : **Preparation of working standard solutions from ascorbic acid stock solution.**

5.6. Quantification of Total Phenols :

The total phenolic content was determined by Folin-Ciocalteu method. Briefly, 20 µl of each plant extract was diluted to 2ml by dissolving in distilled water and each sample was taken in duplicates. To each test tube, 200 µl of Folin-Ciocalteu's reagent (FCR) was added and mixed thoroughly. It was followed by the addition of 500 µl of 20% Na_2CO_3 solution (prepared by dissolving 20 gm of Na_2CO_3 in 100 ml of distilled water) and kept at 55˚C for one hour in water bath. The absorbance was recorded at 760 nm using UV-Visible spectrophotometer. The distilled water was taken as a blank.

5.6.1. Preparation of Standard :

A standard solution of Gallic acid was prepared with the known concentration of 0.1mg/ml (prepared by dissolving 10 mg of Gallic acid powder in 100 ml of distilled water). The five different concentrations of this standard solution were prepared (20µl- 100µl) and taken in duplicates as shown in *table 2*. To each test tube the solutions of 200 µl of Folin-Ciocalteu (FCR) and 500 µl of 20% Na_2CO_3 were added and incubated in water bath at 55˚C for one hour. The absorbance was measured at 760 nm using UV-Visible spectrophotometer.

5.6.2. Preparation of Extract Dilutions :

The dilutions were prepared by taking 20 µl of each plant extract in duplicates and diluted to 2ml by distilled water. To each test tube, the freshly prepared solutions of 200 µl FCR reagent and 500 µl of 20% Na_2CO_3 were added and mixed thoroughly. All of the tubes were incubated at 55˚C in water bath for one hour and the absorbance was measured at 760 nm using UV-Visible spectrophotometer.

S. No.	**Gallic Acid**	**Distilled Water**	**FCR Reagent**	**Na_2CO_3**
S1	20 µl	1980 µl	200 µl	500 µl
S2	40 µl	1960 µl	200 µl	500 µl
S3	60 µl	1940 µl	200 µl	500 µl
S4	80 µl	1920 µl	200 µl	500 µl
S5	100 µl	1900 µl	200 µl	500 µl

Table 2 : **Preparation of working standard solutions from Gallic acid stock solution**.

5.7. Quantification of Total Flavonoids :

The total flavonoids were estimated by aluminium chloride colorimetric assay. Briefly, 20 µl of each plant extract sample was diluted to 2 ml by distilled water and taken in duplicates. To each of test tube, freshly prepared solutions of 100 µl each of 10% $AlCl_3$ (prepared by dissolving 10 gm aluminium chloride in 100 ml distilled water) and 1M KCH_3COO were added to all tubes, covered with silver foil and incubated at room temperature for half an hour. The absorbance was recorded at 470 nm using UV-Visible spectrophotometer. Distilled water was taken as a blank.

5.7.1. Preparation of Standard :

The standard solution for flavonoid estimation, Quercitin was prepared with the known concentration of 0.1 mg/ml (prepared by dissolving 10 mg of quercitin powder in 100 ml distilled water). The five different concentrations of this standard solution were prepared (20 µl- 100 µl) and taken in duplicates as shown in *table 3*. Freshly prepared solutions of 100 µl each of 10% $AlCl_3$ and 1M KCH_3COO were added to all tubes kept at room temperature for half an hour. The absorbance was measured at 470 nm using UV-Visible spectrophotometer.

5.7.2. Preparation of Extract Dilutions :

20 µl of each plant extract were taken in duplicates and diluted to 2ml by distilled water. To each test tube, 100 µl each of 10% $AlCl_3$ and 1M KCH_3COO were added and kept at room temperature for half an hour and recording absorbance at 470 nm using UV-Visible spectrophotometer.

S. No.	Quercitin	Distilled Water	10% $AlCl_3$	1M KCH_3COO
S1	20 µl	1980 µl	100 µl	100 µl
S2	40 µl	1960 µl	100 µl	100 µl
S3	60 µl	1940 µl	100 µl	100 µl
S4	80 µl	1920 µl	100 µl	100 µl
S5	100 µl	1900 µl	100 µl	100 µl

Table 3 : Preparation of working standard solutions from Quercitin stock solution.

5.8. Determination of Antimicrobial Potential :

The antimicrobial potential of plant extracts was carried out against four pathogenic strains of bacteria, namely, *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* by disc diffusion method on nutrient agar media using streptomycin sulphate as positive control (prepared by dissolving). The agar plates were prepared in duplicates and to each plate the bacterial cultures were spread plated using sterile glass spreader. The sterile discs were put on agar plates and to each disc 50µl of crude plant extract was added carefully under sterile conditions of laminar air flow. The plates were incubated overnight at 37˚C in incubator and presence of zone of inhibition (mm) indicated the antimicrobial activity.

S. No.	Strains	Plant Extract	Positive Control
S1	*E.coli*	50 µl	Streptomycin
S2	*B. subtilis*	50 µl	Streptomycin
S3	*S. aureus*	50 µl	Streptomycin
S4	*P. aeruginosa*	50 µl	Streptomycin

Table 4 : **Antimicrobial Potential Determination of plant extracts of *Cichorium intybus* and *Matricaria chamomilla*.**

CHAPTER 6

RESULTS AND DISCUSSION

6.1. Phytochemical Analysis :

The stem and leaves of both these plants were screened for presence or absence of different phytochemicals. The plant parts were studied in the aqueous extracts (Table 5). Flavonoids, phenols, saponins, steroids, tannins, glycosides, and terpenoids are present in both the plant extracts but they differ in their amount. In case of *Matricaria chamomilla*, the phenols, flavonoids, and glycosides are present abundantly in leaves, while as in stem extract, flavonoids, phenol, and saponins are present in higher amounts. Similarly, saponins and tannins are mildly present in leaves. Alkaloids and gums and mucilage were absent in leave extracts of the above plant. Terpenoids were found to be in trace amounts in both stem and leaves. Steroids were absent in stem extracts while as tannins were mildly present.

In case of *Chicory intybus*, flavonoids, steroids, tannins, and terpenoids were present abundantly in leaves. Phenols were mildly present, and saponins were found in trace amounts. Similarly, in stem extracts flavonoids, phenols, saponins, and terpenoids were present abundantly, while as steroids and tannins were mildly found.

Phytochemicals (*Chicory intybus*)	**Aqueous leave Extract**	**Aqueous stem Extract**
Alkaloids	_	_
Carbohydrates	++	+++
Flavonoids	+++	+++
Glycosides	+	++
Gums and mucilage	_	_
Phenolics	++	+++
Phlobatannins	++	++
Reducing sugars	+++	++
Saponins	±	+++
Steroids	+++	++
Tannins	+++	++
Terpenoids	+++	+++

Table 5 : Phytochemical evaluation of aqueous extracts of *Chicory intybus.*

Phytochemicals (*Matricaria chamomilla*)	**Aqueous leave Extract**	**Aqueous stem Extract**
Alkaloids	_	+
Carbohydrates	++	+++
Flavonoids	+++	+++
Glycosides	+++	++
Gums and mucilage	_	_
Phenolics	+++	+++
Phlobatannins	++	++
Reducing sugars	+++	+++
Saponins	+	+++
Steroids	+++	_
Tannins	++	++
Terpenoids	±	±

Table 6 : **Phytochemical screening of aqueous extracts of *Matricaria chamomilla*.**

Phytochemicals	**Aqueous leave extract (*C. intybus*)**	**Aqueous leave extract (*M. chamomilla*)**
Alkaloids	Absent	Absent
Carbohydrates	Present moderately	Present moderately
Flavonoids	Present in abundance	Present in abundance
Glycosides	Present in traces	Present in abundance
Gums and mucilage	Absent	Absent
Phenolics	Present moderately	Present in abundance
Phlobatannins	Present moderately	Present moderately
Reducing sugars	Present in abundance	Present in abundance
Saponins	Present in traces	Present moderately
Steroids	Present in abundance	Present in abundance
Tannins	Present in abundance	Present moderately
Terpenoids	Present in abundance	Present in traces

Table 7 : Comparison of phytochemicals in leave extracts of *Chicory intybus* and *Matricaria chamomilla.*

Phytochemicals	**Aqueous stem extract (*C. intybus*)**	**Aqueous stem extract (*M. chamomilla*)**
Alkaloids	Absent	Present in traces
Carbohydrates	Present in abundance	Present in abundance
Flavonoids	Present in abundance	Present in abundance
Glycosides	Present moderately	Present moderately
Gums and mucilage	Absent	Absent
Phenolics	Present in abundance	Present in abundance
Phlobatannins	Present moderately	Present moderately
Reducing sugars	Present moderately	Present in abundance
Saponins	Present in abundance	Present in abundance
Steroids	Present moderately	Absent
Tannins	Present moderately	Present moderately
Terpenoids	Present in abundance	Present in traces

Table 8 : **Comparison of phytochemicals in stem extracts of *Chicory intybus* and *Matricaria chamomilla.***

6.2. DPPH Free Radical Scavenging Activity :

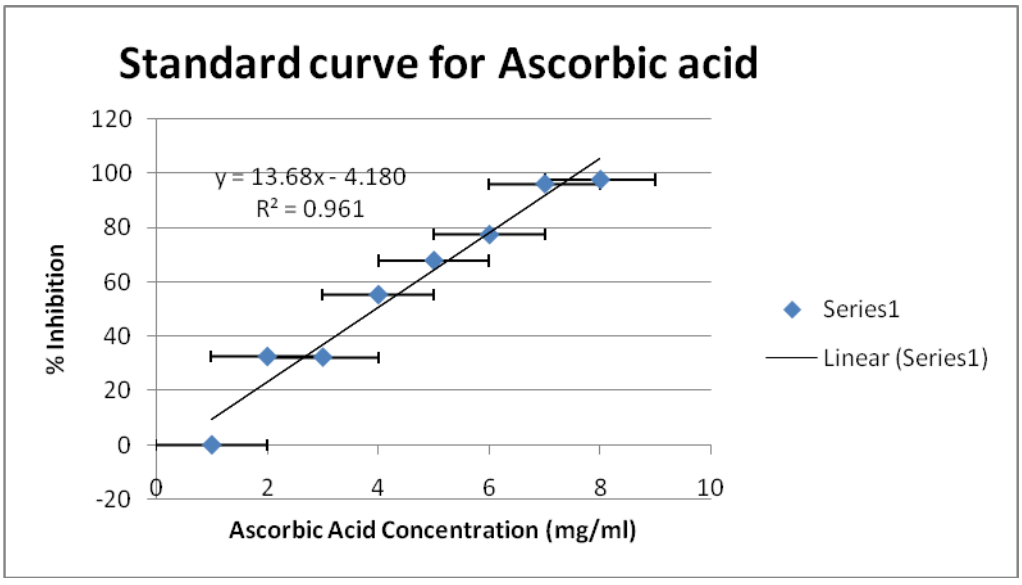

Figure 4 : Graph showing *Ascorbic acid concentration* (mg/ml) vs *% inhibition*. The absorbance was recorded at 517 nm using UV-Visible spectrophotometer. All values are expressed as Mean ± SEM (n=3).

Eight different plant aqueous extract concentrations (20µl- 160µl) were used to assess the antioxidant potential of stem and leaves of *Cichorium intybus* and *Matricaria chamomilla*. The

aqueous extracts of chicory stem and leaves exhibited maximum antioxidant activity with 50% inhibition (IC_{50}) at 6.01 ± 0.28µg/ml and 3.52 ± 0.35µg/ml than aqueous extarcts of chamomile with (IC_{50}) values of 18.89 ± 0.27µg/ml and 23.90 ± 0.48µg/ml respectively as shown in *figure 5*.

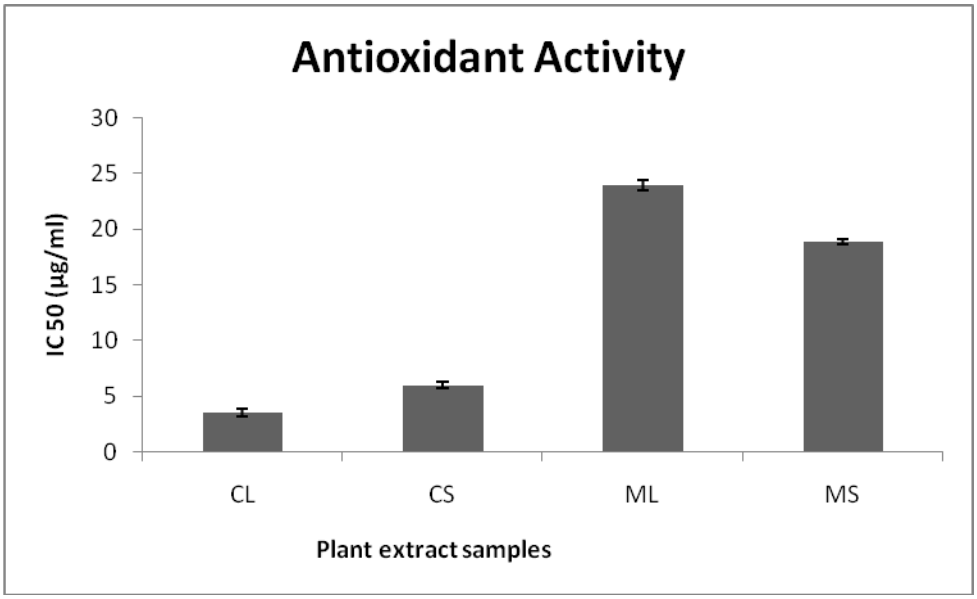

Figure 5 : Graph showing *IC_{50} values* (vs) *plant samples*. Antioxidant activity of plant aqueous extracts. *CL*- Chicory leaf; *CS*- Chicory Stem; *ML*- Matricaria leaf; *MS*- Matricaria stem. All values are expressed as Mean ± SEM (n=3).

In addition to the *IC_{50}* values of each plant extract, the % inhibition of leaf and stem extracts of both the plants were evaluated separately as shown in the *figure 6,7,8* and *9*.

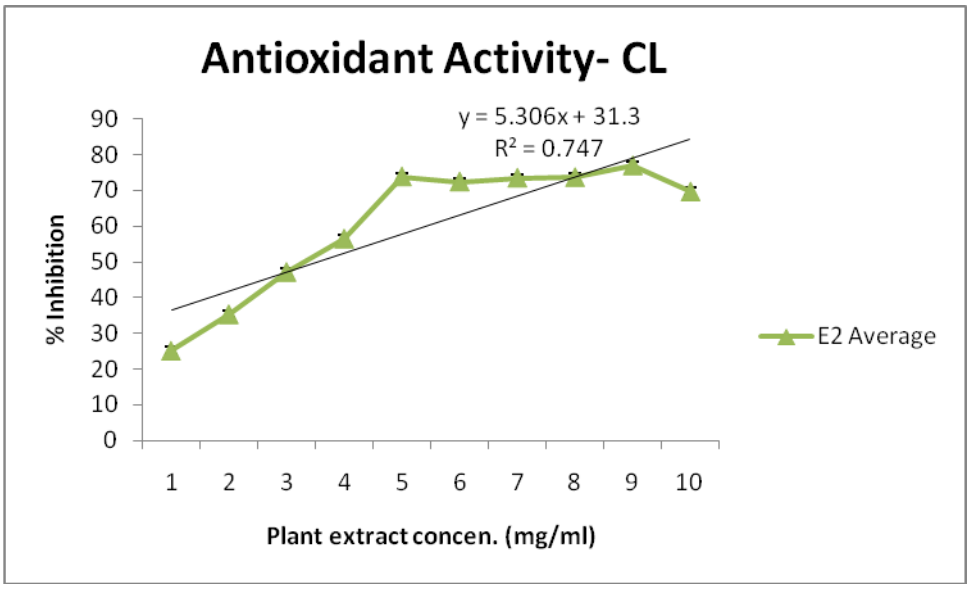

Figure 6 : Graph showing *plant extract concentration* (vs) *% inhibition* of chicory leaf extract (CL). All values are expressed as Mean ± SEM (n=3).

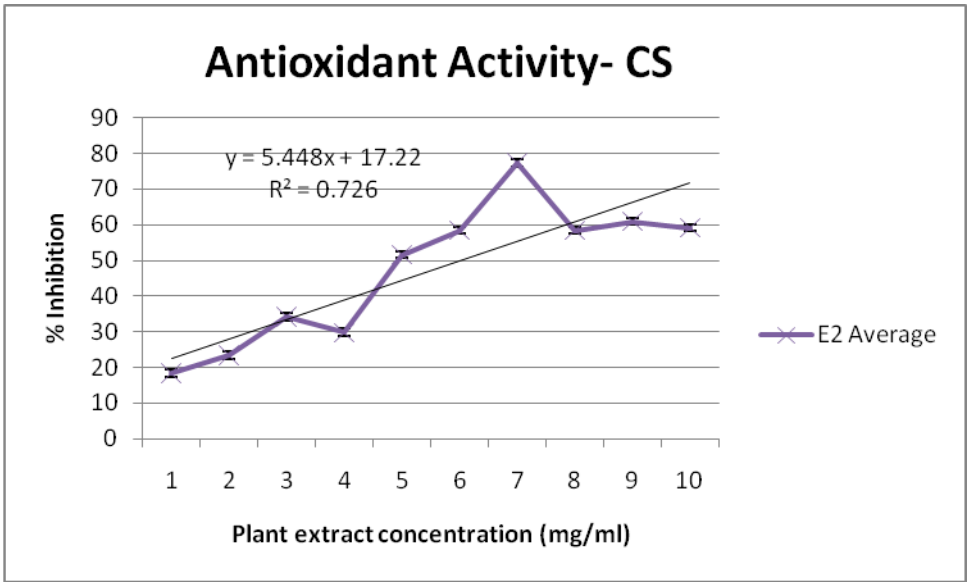

Figure 7 : Graph showing *plant extract concentration* (vs) *% inhibition* of chicory stem extract (CS). All values are expressed as Mean ± SEM (n=3).

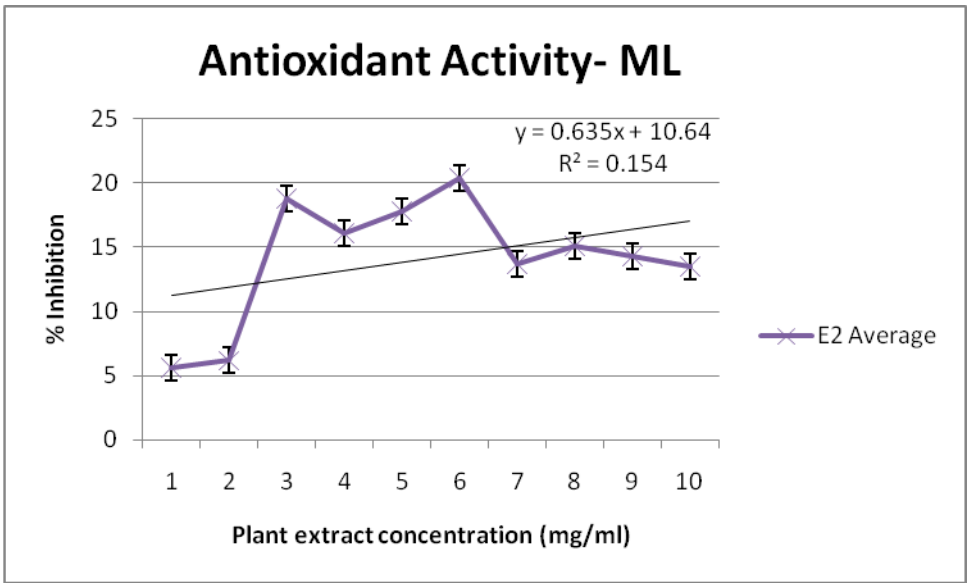

Figure 8 : Graph showing plant extract concentration (vs) % inhibition of Matricaria leaf extract (ML). All values are expressed as Mean ± SEM (n=3).

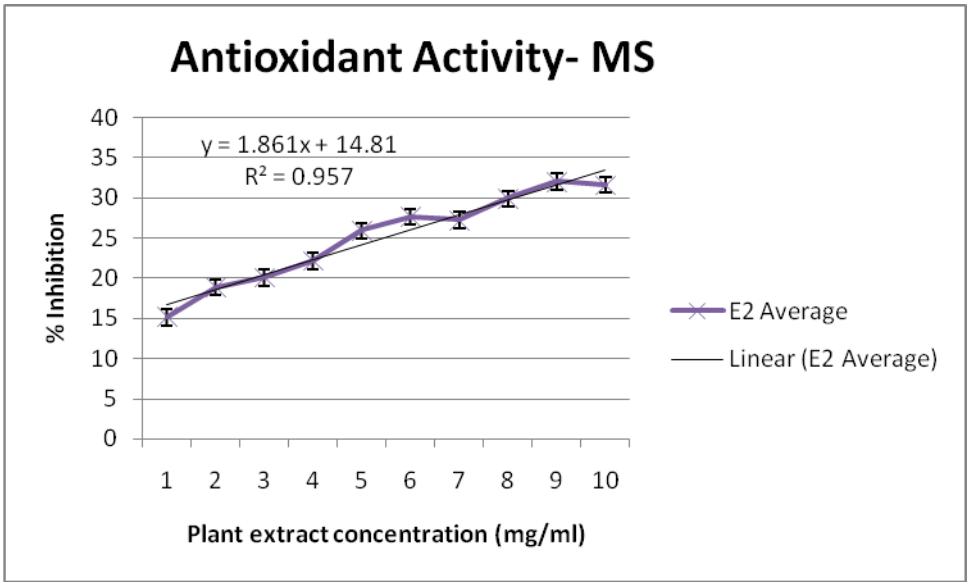

Figure 9 : Graph showing *plant extract concentration* (vs) *% inhibition* of Matricaria stem extract (MS). All values are expressed as Mean ± SEM (n=3).

6.3. Quantification of Total Phenols :

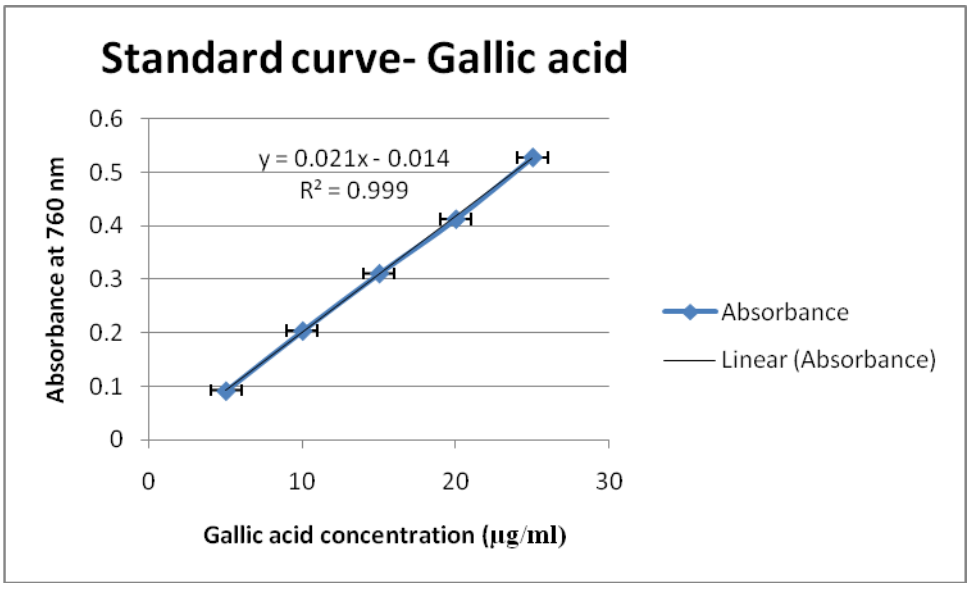

Figure 10 : Graph showing *Gallic acid concentration* (vs) *Absorbance* at 760 nm. Absorbance was recorded at 760 nm using UV-Visible spectrophotometer. All values are expressed as Mean ± SEM (n=3).

20µl of each plant extract sample was analyzed for the total phenolic content using Gallic acid as standard. The aqueous extracts of chicory stem and chamomile leaf exhibited higher phenolic content with concentration of 19.77 ± 0.20µg/ml and 19.28 ± 0.87 respectively than chicory leaf

and chamomile stem with concentration of 10.8 ± 0.12µg/ml and 10.47 ± 0.46µg/ml respectively as shown in *figure 7*.

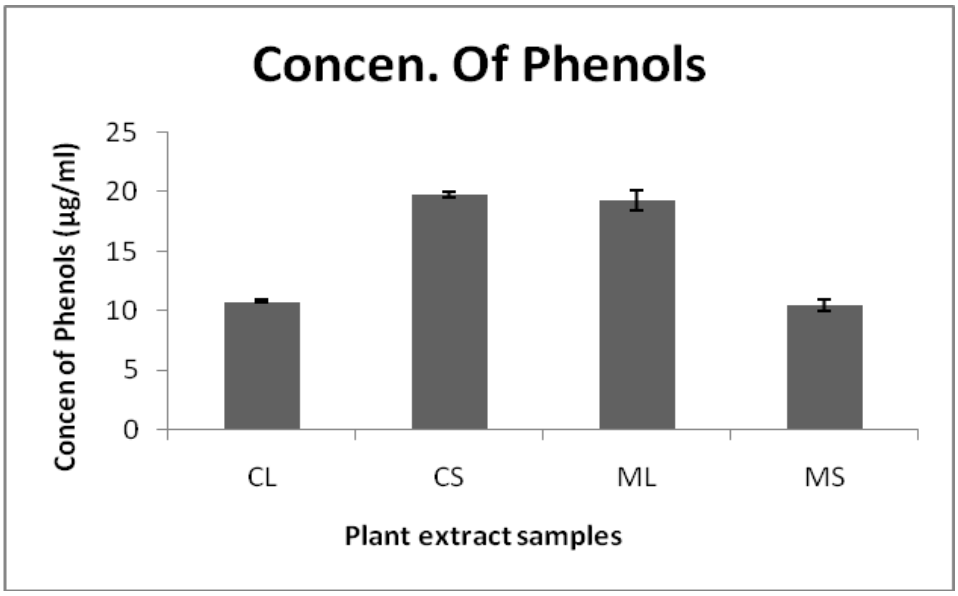

Figure 11 : Graph showing *phenol concentration* (vs) *plant extract samples*. *CL*- Chicory leaf; *CS*- Chicory stem; *ML*- Matricaria leaf; *MS*- Matricaria stem. All values are expressed as Mean ± SEM (n=3).

The total phenolic content in the plant extracts were evaluated using gallic acid as standard and expressed as µg/ml of gallic acid concentration as shown in the *table 9*.

S.NO.	Plant Extracts	Total Phenolic Content (TPC) µg/ml of Gallic acid
1.	Chicory Leaf (CL)	10.8±0.12
2.	Chicory Stem (CS)	19.77±0.20
3.	Matricaria Leaf (ML)	19.28±0.87
4.	Matricaria Stem (MS)	10.47±0.46

Table 9 : **Total Phenolic content (TPC) of plant extracts. All values are expressed as Mean ± SEM (n=3).**

6.4. Quantification of Total Flavonoids:

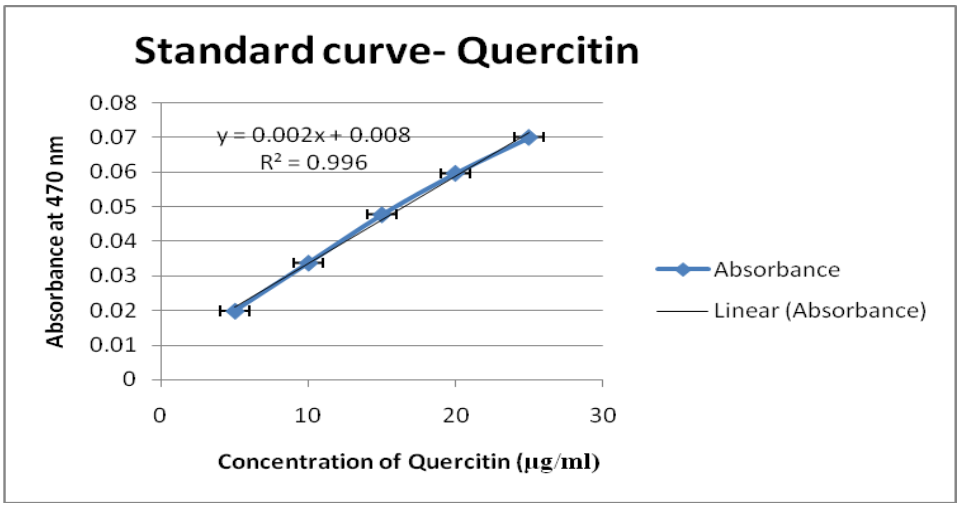

Figure 12 : Graph showing *Quercitin concentration* (vs) *Absorbance* at 470 nm. The absorbance was recorded using UV-Visible spectrophotometer. All values are expressed as Mean ± SEM (n=3).

The plant aqueous extracts of stem and leaves of chicory and chamomile were evaluated for the presence of total flavonoids. The aqueous extracts of chicory leaf and stem showed higher quantities of flavonoids with the concentration of 18.36 ± 0.32µg/ml and 17.24±0.48µg/ml respectively than aqueous extracts of chamomile leaf and stem with the concentration of 11.5 ± 0.56µg/ml and 7.76 ± 0.32µg/ml respectively as shown in *figure 9*.

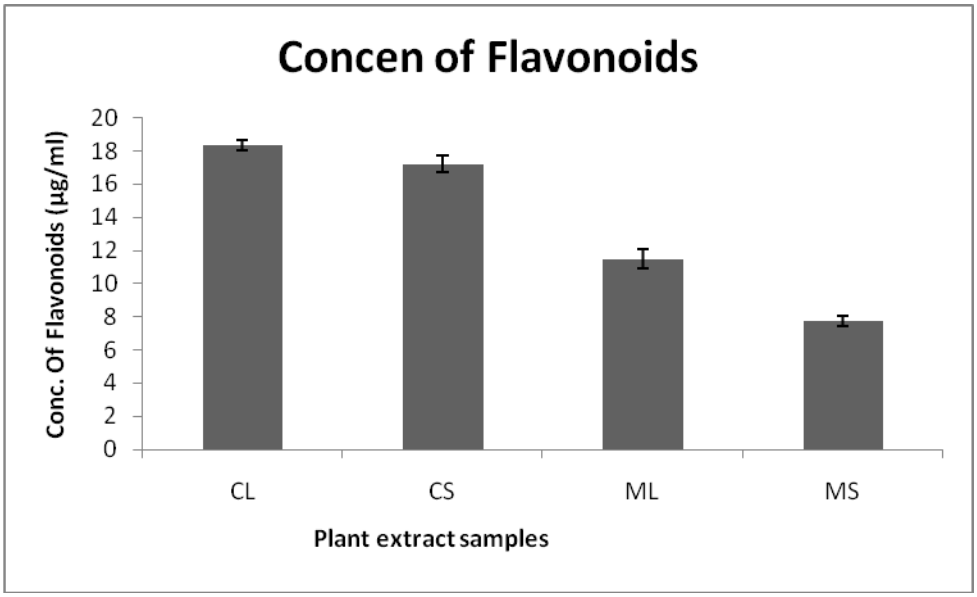

Figure 13 : Graph showing *flavonoid concentration* (vs) *plant extract samples*. *CL*- Chicory leaf; *CS*- Chicory stem; *ML*- Matricaria leaf; *MS*- Matricaria stem. All values are expressed as Mean ± SEM (n=3).

The total flavonoid content was evaluated using Quercitin as standard and the values were expressed as µg/ml of quercitin in the respective plant extract samples as shown in the *table 10*.

S.NO.	Plant Extracts	Total Flavonoid (TFC) (µg/ml) of Quercitin
1.	Chicory Leaf (CL)	18.36±0.32
2.	Chicory Stem (CS)	17.24±0.48
3.	Matricaria Leaf (ML)	11.5±0.56
4.	Matricaria Stem (MS)	7.76±0.32

Table 10 : **Total flavonoid concentration (TFC) of plant extracts. All values are expressed as Mean ± SEM (n=3).**

6.5. Antimicrobial Potential of Extracts :

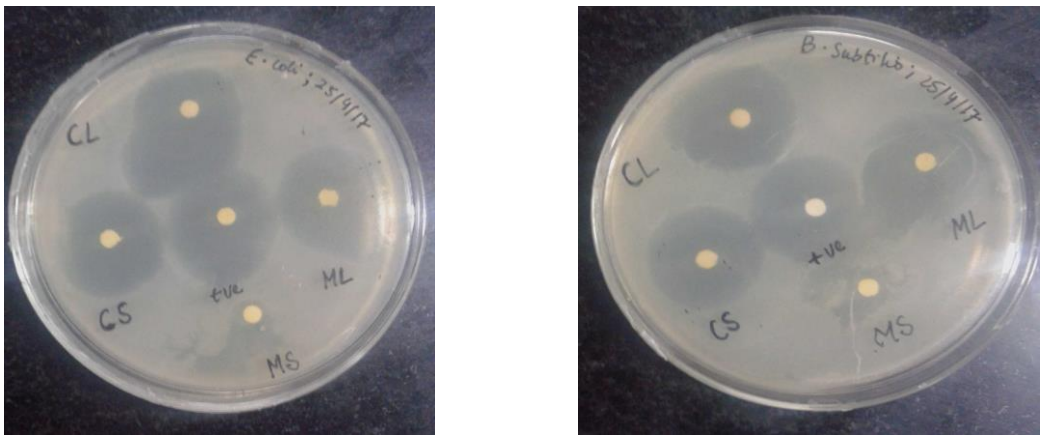

Figure 14 : Antimicrobial potential of plant extracts showing zones of inhibition.

A- *Escherichia.coli*. **B**- *Bacillus subtilis*; *CL*- Chicory leaf; *CS*- Chicory stem;

ML- Matricaria leaf*; MS*- Matricaria stem.

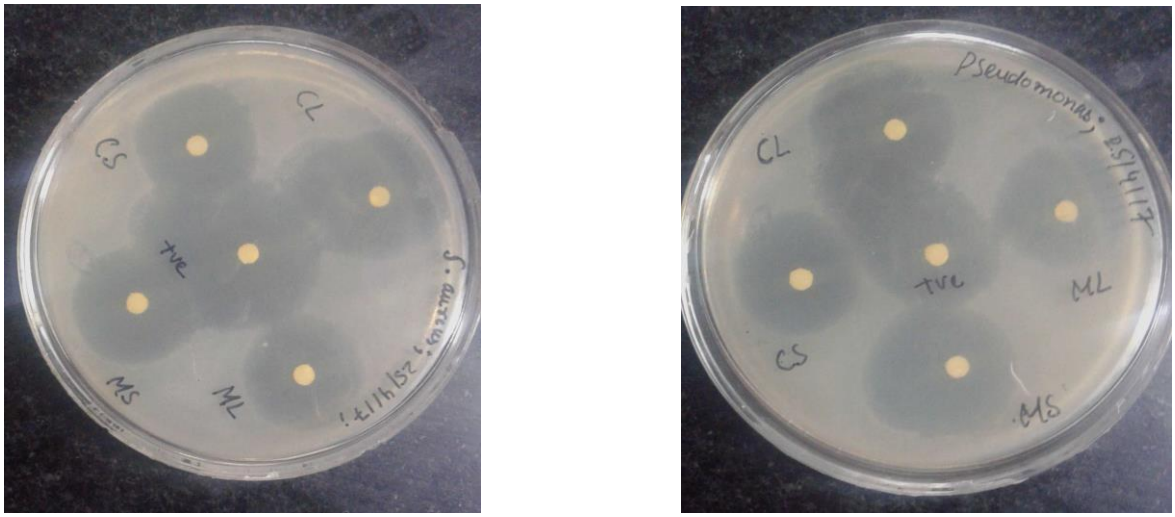

Figure 15 : Antimicrobial potential of plant extracts showing zones of inhibition.

. **A**- *Staphylococcus.aureus*. **B**- *Pseudomonas aeruginosa*; *CL*- Chicory leaf; *CS*- Chicory stem; *ML*- Matricaria leaf; *MS*- Matricaria stem.

The plant extracts were evaluated for their antimicrobial potential against four pathogenic bacteria, namely, *E.coli, S.aureus, P.aeruginosa* and *B.subtilis* using disc diffusion method. Antibiotic, streptomycin sulphate with the concentration of 0.025 gm/ml was used as a positive control (prepared by dissolving 0.125 gm/5ml of distilled water). The aqueous extracts of chicory leaf and stem showed maximum zone of inhibition against all four pathogenic strains with 25.5±028 mm *(E.coli*), 25.8±0.08 mm (*P.aeruginosa*), 28.7±0.14 mm (*S.aureus*), and 26.2±0.1 mm *(B.subtilis*) for leaf extracts and 23.9±0.05 mm *(E.coli*), 22.6±0.3 mm (*P.aeruginosa*), 24.6±0.3 mm (*S.aureus*), and 24.7±0.1 mm (*B.subtilis*) for stem extracts as shown in the *table 9*. All of the experiments were carried out in triplicates.

In case of aqueous extracts of chamomile, the stem extract was less effective against two pathogenic strains- *E.coli* and *B.subtilis*, however, leaf extract showed significant antimicrobial activity against all four strains. The zones of inhibition for leaf and stem extracts were 23.7±0.4 mm (*E.coli*), 24.9±0.05 mm (*P.aeruginosa*), 21.8±0.1 mm *(S.aureus*), and 23.9±0.05 mm (*B.subtilis*), and for stem extract the zones of inhibition were measured to be 9.9±0.05 mm (*E.coli*), 27.4±0.3 mm (*P.aeruginosa*), 22.7±0.4 mm (*S.aureus*), and 9.2±0.1 mm (*B.subtilis*). The zones of inhibition were measured in diameter to assess the antimicrobial potential of plant

extracts. It can be concluded from the results that these two plant extracts possess significant antimicrobial activity.

S.NO.	Strains	Zones of Inhibition (mm)			
		Chicory Leaf (CL)	**Chicory Stem (CS)**	**Matricaria Leaf (ML)**	**Matricaria Stem (MS)**
1.	*E.coli*	25.5±0.28	23.9±0.05	23.7±0.40	9.9±0.05
2.	*P.aeruginosa*	25.8±0.08	22.6±0.3	24.9±0.05	27.4±0.3
3.	*S.aureus*	28.7±0.14	24.6±0.3	21.8±0.1	22.7±0.4
4.	*B.subtilis*	26.2±0.1	24.7±0.1	23.9±0.05	9.2±0.1

Table 11 : **Antimicrobial activity of plant extracts showing zones of inhibition (mm) against four pathogenic microorganisms**. **All values are expressed as Mean ± SEM (n=3).**

CHAPTER 7

CONCLUSION

The plant parts of the *Chicory intybus* and *Matricaria chamomilla* are used as herbal medicines for various diseases. The plant parts have been characterized on the basis of their morphological parameters. The presence of various secondary metabolites like flavonoids, glycosides, steroids, tannins, carbohydrates and terpenoids makes these plants medicinally very important. The *Chicory intybus* leaves are eaten raw in salads in winter as it has digestive properties and helps to cure different digestive disorders. Therefore, these plant parts and their extract can be used in the preparation of different alternative medicines for various kinds of diseases. For this purpose, detailed study of these plants are required to investigate their all medicinal properties as their functions are still largely unknown.

CHAPTER 8

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APPENDIX

CHEMICALS

NaOH: Sodium hydroxide

H_2SO_4: Sulphuric acid

KOH: Potassium hydroxide

HCl : Hydrochloric acid

$FeCl_3$: Ferric chloride

COOH: Carboxylic acid

TERMS

Antibacterial: Potential to kill or inhibit the growth of bacteria.

Antifungal : Potential to kill or inhibit the growth of fungi.

Antioxidant: Potential to prevent oxidation of macromolecules by scavenging free radicals produced in body.

Anti-ulcerogenic: Potential to prevent formation of ulcers.

Antiseptic: Potential to prevent growth of all disease-causing microorganisms.

Anti-inflammatory: Potential to reduce the inflammation or swelling.

Antispasmodic: Potential to relieve or prevent the incidence of spasm in the muscles.

Sudorific: Potential to cause or increase sweating.

Sedative: Potential to induce sleep and calm.

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