QUANTIFICATION OF BIOACTIVE MOLECULES AND EVALUATION OF ANTI-MICROBIAL POTENTIAL OF *Tinospora cordifolia* Miers

Thesis

by

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(NH-2019-26-M)

submitted to

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CERTIFICATE- I

This is to certify that the thesis titled **"Quantification of bioactive molecules and evaluation of anti-microbial potential of *Tinospora cordifolia* Miers"**, submitted in partial fulfilment of the requirements for the award of degree of **MASTER OF SCIENCE, MOLECULAR BIOLOGY AND BIOTECHNOLOGY** in the discipline of **BIOTECHNOLOGY** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, (Nauni) Solan (HP) – 173230 India is a record of bonafide research work carried out by **Mr. Ashish Kumar Rajvanshi (NH-2019- 26-M)** son of **Shri Kulbir Kumar** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigation has been fully acknowledged.

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CERTIFICATE- II

This is to certify that the thesis titled **"Quantification of bioactive molecules and evaluation of anti-microbial potential of *Tinospora cordifolia* Miers"**, submitted by **Mr. Ashish Kumar Rajvanshi (NH-2019-26-M)** son of Shri. Kulbir Kumar to the Dr. Yashwant Singh Parmar University of Horticulture & Forestry, (Nauni) Solan (HP) – 173230 India in the partial fulfilment for the requirements for the degree of **MASTER OF SCIENCE** in the discipline of **BIOTECHNOLOGY** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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This is to certify that all the mistakes and errors as pointed out by the external examiner have been incorporated in the thesis titled, **"Quantification of bioactive molecules and evaluation of anti-microbial potential of *Tinospora cordifolia* Miers"**, submitted by **Mr. Ashish Kumar Rajvanshi (NH-2019-26-M)** son of Shri. Kulbir Kumar to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, (Nauni) Solan (H.P.)- 173230 in the partial fulfillment for the award of degree of **MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY**.

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Place: Neri, Hamirpur

Dated: **(Ashish Kumar Rajvanshi)**

CONTENTS

ABBREVIATIONS USED

%	:	Percent
^{o}C	:	Degree Celsius
CD	:	Critical Difference
SE	:	Standard Error
H.P.	:	Himachal Pradesh
UHF	:	University of Horticulture and Forestry
COH&F	:	College of Horticulture and Forestry
mm	:	Millimeter
cm	:	Centimeter
et al.	:	And co-worker/ and others
Fig	:	Figure
Min	:	Minute
Sec	:	Second
Hr	:	Hour
L	:	Litre
RW	:	Root Water
SW	:	Stem Water
LW	:	Leave Water
RE	:	Root Ethanol
SE	:	Stem Ethanol
LE	:	Leave Ethanol
RM	:	Root Methanol
SM	:	Stem Methanol
LM	:	Leave Methanol
RC	:	Root Chloroform
SC	:	Stem Chloroform
LC	:	Leave Chloroform
g	:	Gram
i.e.	:	That is
m	:	Meter
mg	:	Milligram
CRD	:	Completely Randomized Design
ml	:	Milliliter
mm	:	Millimeter
µl	:	Micro liter
µm	:	Micro meter
/	:	Per

sp.	:	Species
mg/ml	:	Milligram per mililitre
mg/100g	:	Milligram per hundred gram
ANOVA	:	Analysis of Variance
TLC	:	Thin Layer Chromatography
HPTLC	:	High Performance Thin Layer Chromatography
LC-MS	:	Liquid Chromatography – Mass Spectrometry
°C	:	Degree Celesius
MIC	:	Minimum Inhibitory Concenteration
MBC	:	Minimum Bactericidal Concenteration
LC – MS	:	Liquid Chromatography – Mass Sperctrometry

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Chapter – 1

Introduction

Medicinal plants have been used as natural medicines. This kind of activities had been in existence since pre-historic times. In India, most of the population depends on the conventional type of medicine (Achary and Shrivastava, 2008). Indian folk-medicine and ethnobotany had included 2532 plant species and many of which have medicinal properties (Jain, 1994). Due to rise in side effects of unfavorable drug reactions, the interests of government and academics in conventional medicines are growing rapidly. Plants have formed the basis of worldly traditional medicine systems that have been in existence for thousands of years and continue to provide new improvements for humanity. In today's world natural products and their derivatives represent more than 50% of all the drugs which are useful for clinical purposes (Kean *et al.* 2016). Medicinal plants are used as source for great economic value all over the world. Natural products are important sources for biologically active drugs. In India for thousands of years, it is believed to be existence of Ayurvedic form of medicine (Krishnaiah *et al.* 2009).

Among plants of economic importance, medicinal and aromatic plants have played a vital role in alleviating human sufferings. Plants are utilized as therapeutic agents since time immemorial in both organized (Ayurveda, Unani) & unorganized (folk, tribal, native) forms. Demand for medicinal plants is increasing in both developing and developed countries. Research on medicinal plants is one of the leading areas of research globally. Uses of medicinal plants in the industrialized societies have been traced from the extraction and development of several drugs and chemotherapeutic drugs from these plants as well as from traditionally used rural herbal remedies. Among the vast library of important medicinal plants *Tinospora cordifolia* Miers of family Menispermeaceae is immensely valuable in terms of chemical constituents and in pharmacology. The plant family Menispermeaceae consists of about 70 genus and 450 species that are found in tropical low land regions. They are generally climbing or twinning rarely shrubs. Leaves are alternate or lobed, flowers are small cymose, seeds are usually hooked or reniform. This family is rich source of alkaloids and terpenes. *Tinospora* is one of the important genera of the family, consisting of about 15 species and some medicinally important species includes *T. cordifolia, T. malabarica, T. tomentosa, T. crispa, T. uliginosa* etc. (Raghu *et al.* 2006).

Tinospora cordifolia is one of the non - controversial and extensively used herb in

ayurvedic medicine. The common name of *Tinospora cordifolia* in Sanskrit is Guduchi which belongs to Menispermaceae family. It is a large, deciduous shrub with greenish yellow typical flowers. It is typically found at higher altitudes (Rana *et al.* 2012). *Tinospora cordifolia* is known by different name in various different languages in India *viz.*, Tippa-teega (Telugu), Shindilakodi (Tamil), Amruthu, Chittamruthu (Malayalam), Amrutha balli (Kannada), Rasakinda (Sinhala), Gurcha (Hindi), Garo (Gujarati), Amritavalli (Sanskrit), Guduchi (Marathi) (Gaur *et al.* 2014).

Tinospora is a climber distributed throughout tropical and subtropical India and only three species of the plant are found in the Indian subcontinent. The three species include: *Tinospora cordifolia* Miers, *Tinospora malabarica* Miers, and *Tinospora crispa* Miers. *Tinospora cordifolia* is widely distributed throughout all the tropical regions of the country. However, in the North-western regions especially Jammu and Himachal Pradesh, it is in abundance. Unlike the wide distribution of *Tinospora cordifolia*, the other two species of the plant (*Tinospora malabarica* and *Tinospora crispa*) are distributed over limited areas. *Tinospora malabarica* is found in the southern Kerala region of the country and *Tinospora crispa* grows only in North-eastern Himalayas of Assam.

Botanical Descriptions

Tinospora cordifolia is a deciduous and subsatainly climbing shrub having multiple elongated twining branches. The leaves are simple, alternate, having long petioles up to 14 cm pulvinate, roundish, both at the base and apex with the basal one longer and twisted partially and half way around. The flowers of *Tinospora cordifolia* are unisexual, small and greenish yellow on axillary and terminal racemes. Sepals 6, free in two series of three each, the outer ones are smaller than the inner. Petals 6 free smaller than sepals, obovate and membranous.

The Taxonomical description of this medicinal herb is given below;

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Ranunculales
Family	:	Menispermaceae
Genus	:	Tinospora
Species	:	Cordifolia

Habitat: *Tinospora cordifolia* prefers wide range of soil, acid to alkaline and it needs moderate level of soil moisture. Found throughout tropical India, ascending to an altitude of 1000 feet and in South Asia, Indonesia, Phillipians, Thailand, Myanmar, China and in Srilanka worldwide.

Chemical Composition: A mixture of constituents have been isolated from roots, stem and leaves of *Tinospora cordifolia*. The constituent belongs to different classes such as diterpenoid lactones, alkaloids glycosides aliphatic compounds, steroids, polysaccharides. Certain constituents isolated from plant belongs to tinosporic acid, cordifolisides A to E, syringen, berberine, giloin, tinosporone crude giloininand, gilenin, tinosporide, arabinogalactan polysaccharide, picrotene, gilosterol, tinosporol, tinosporidine, sitosterol, cordifol, heptacosanol, octacosonal, columbin, bergenin, chasmanthin, palmarin, palmatosides C and F, tetrahydropalmaitine, isocolumbin, amritosides, cordioside, magnoflorine, tinosponone, ecdysterone, makisterone A, hydroxyecdysone, tembetarine, syringine, glucan polysaccharide, syringine apiosylglycoside, palmatine, jatrorrhizine, respectively (Singh *et al*. 2003).

Fig. 1.1 – Leaves and stems of *Tinospora cordifolia*

Importance

Tinospora cordifolia is a prime drug of Indian systems of medicine and is widely used for its medicinal properties. The drug is well known Indian bitter and prescribed in fevers, diabetes, dyspepsia, jaundice, fevers, diabetes, urinary problems, chronic and skin diseases diarrhoea and dysentery. It has been also indicated useful in the treatment of heart disease, leprosy, and helmenthiasis. The starch obtained from the stem is highly nutritive and digestive and used in many diseases (Sinha *et al. 2004*). The stem of the plant is one of the main constituents of

several Ayurvedic/herbal preparations that are used in general debility, dyspepsia, fever. (Nayampalli *et al.* 1988). The extract of the plant stem is useful in the management of skin disorders (Aiyer *et al.* 1963). Dry bark of *Tinospora cordifolia* has anti-spasmodic, antipyretic, anti-allergic, anti-inflammatory, and antileprotic properties (Ikram *et al.* 1987, Asthana *et al.* 2001).

A variety of active components derived from the plant like aliphatic, alkaloids, steroids, diterpenoid lactones, alkaloids, steroidsal, and glycosides have been isolated from the different parts of the plant body, including root, stem, and whole plant (Upadhyay *et al*. 2010). Recently, the plant is of great interest to researchers across the globe because of its reported medicinal properties like anti-cancer, anti-oxidant, anti-inflammatory, anti-oxidant, anti-spasmodic, anti-malarial, immunoprotective, immunomodulatory and anti- tuberculosis activities (Ansari *et al.* 2016; Shanthi and Nelson, 2013).

These compounds have been reported to have different biological roles in disease conditions thus enabling potential application in clinical research. *Tinospora cordifolia* extracts are extensively used in various herbal preparations for the treatment of different ailments for its anti-periodic, anti-spasmodic, anti-microbial, anti-osteoporotic, anti-inflammatory, anti-arthritic, anti-allergic, and anti-diabetic properties.

The *Tinospora cordifolia* have antimicrobial activities due to the presence of bioactive molecules in the plant which is not only responsible for medicinal and therapeutic values but also have a dietary and nutritive value. The methanol extracts of *Tinospora cordifolia* have been reported to have potential against microbial infections. The anti-bacterial activity of *Tinospora cordifolia* extracts has been assayed against *Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Salmonella typhi, Shigella flexneri, Salmonella paratyphi, Pseudomonas aeruginosa, and Serratia marcesenses.* (Shah and Ghosh, 2020.)

Market Potential

The annual consumption of the crude drug mostly by Ayurvedic pharmaceuticals/herbal drug manufactures is estimated to be 1000 tonnes. Demand for aqueous giloy extract in the world market is growing due to increasing awareness and supportive research into the use of giloy extracts as anti-oxidant supplement, also used as supplement for improving memory, intellect, detox, liver support and blood purifier. Importers, buyers within the country, processors, traditional practitioners, Ayurvedic and Siddha drug manufacturers throng the markets for procurement of this plant every year. Its domestic as well as export demands are quite large.

It is very important to develop a method for the quantification of its bioactive molecules, so that the exact quantity of a particular molecule can be measured in the different herbal products.

Keeping in view the wide application of *Tinospora cordifolia*, it has been aimed to carry out isolation of bioactive molecule from this plant.

1. Quantification of bioactive molecules of *Tinospora cordifolia*.
2. Evaluation of anti-microbial potential of *Tinospora cordifolia.*

Chapter 2
Review of literature

The present study focusses on the quantification of bioactive molecules and evaluation of antimicrobial potential of *Tinospora cordifolia*. The different classes of compounds which are found in this plant are grouped like alkaloids, steroids, terpenoids, polysaccharides, glycosides and different aromatic and aliphatic compounds that are present in their photoactive form that are responsible for the wide range of medicinal and therapeutic properties. The literature on quantification of bioactive molecules and antimicrobial potential is reviewed in the light of available literature:

I) Quantification of bioactive molecules of *Tinospora cordifolia*.

Ahmed *et al.* (2006) studied the quantitative determination of four constituents of *Tinospora* spp. by a reversed-phase HPLC–UV–DAD method. Broad-based studies revealed variation in content of four secondary metabolites in the plant from different eco-geographical regions of India and also describe the separation and quantification of important markers, such as 20β-hydroxyecdysone, tinosporaside, cordioside, and columbin, present in three species of *Tinospora viz., Tinospora cordifolia, T. malabrica,* and *T. crispa.* The studies revealed that the maximum amount of the marker compounds is present in *Tinospora cordifolia* species, especially from accessions collected from higher altitudes of the Jammu province (North India).

Srinivasan *et al.* (2008) revealed in their studies that *Tinospora cordifolia* and *Tinospora sinensis* show differences in chemical constituents. The berberine content of two species show marked variation. *Tinospora cordifolia* having three times higher berberine concentrations. On the other hand, *Tinospora sinensis* contain certain compounds that do not occur in *Tinospora cordifolia.*

Sivakumar and Rajan (2011) performed HPLC analysis using petroleum ether, methanol, aqueous and chloroform extracts on using a solvent system of acetonitrile : water (60:40)V/V as mobile phase at a flow rate of 0.5 ml/min and observed that at 265 nm gave good separation of berberine at Rt 5:15min. and maximum content of berberine was observed in methanolic extract as compared to other samples.

Spandana *et al.* (2013) reviewed the importance of the variety of constituents had been

isolated from different parts of *Tinospora cordifolia*. They suggested that the *Tinospora cordifolia* is the best remedy for children suffering from upper respiratory tract infections. The aqueous extract of *Tinospora cordifolia* significantly lowered the serum cholesterol and moves the HDL cholesterol level to basic value. It also possesses antioxidant, anti-hyperglycemic, anti-neoplastic and hepatoprotective properties.

Bala *et al.* (2015) studied a rapid, sensitive, and accurate ultra-performance liquid chromatography coupled with mass spectrometric method (UPLC-MS) for determination of four bioactive compounds, syringin, cordifolioside A, magnoflorine and tinocordiside in the stem of *Tinospora cordifolia*. The variation of these four bioactive compounds in *Tinospora cordifolia* hosted on fifteen different trees was also determined. These compounds were found in high amount in the *Tinospora cordifolia* hosted on *Azadirachta indica* and *Mangifera indica* as compared with other plants and the NMR fingerprinting of the extract revealed the presence of alkaloids, fatty acid methyl esters, polysaccharides and marker components of *Tinospora cordifolia*.

Albinjose *et al.* (2015) investigated the bioactive compounds of *Tinospora cordifolia by* gas chromatography – mass spectrometry (GC-MS) and found that maximum no. of fractions (7) extracted from petroleum ether of extract *Tinospora cordifolia* by column chromatography. The high Rf value compounds obtained from TLC were further subjected to the analysis of HPLC and after that in GC-MS analysis, 40 compounds (chloroform – 15, methanol -14 and petroleum ether -11) were detected in the extract of *Tinospora cordifolia.*

Bala *et al.* (2015) isolated the eight bioactive molecules of *Tinospora cordifolia* by chromatographic purification like (1) N-formylannonain (2) Magnoflorine (3) Jatrorrhizine (4) Palmatine (5) 11-hydroxymustakone (6) cor-difolioside (7) Tinocordiside (8) Yangambin and out of these (1) N-formylannonain (5) 11-hydroxymustakone (8) Yangambin are found in highest amount and concluded that eight compound have been isolated and characterized belonging to different classes. The pharmacological evaluation of extract, fractions, and pure molecules showed the ethnomedicinal value of *Tinospora cordifolia* for anticancer and immunomodulatory activities.

Sonkamble and Kamble (2015) identified *T. cordifolia* phytochemicals as well as compounds with antidiabetic properties in the context of -amylase inhibition. The total phenolic content of *T. cordifolia* extracts varied significantly, with ethanol extract having the highest phenolic content and ethyl acetate extract having the highest -amylase inhibition. Extracts having greater than 40% inhibitory action were evaluated using LCMS and identified using MassBank,

ChemSpider, and the Phenol Explorer database. Investigated for known antidiabetic action in the identified compounds and discovered seven: Cyanidin 3-Osambubiosyl 5-O-glucoside, Hesperetin 7-Rhamnoglucoside, quercetin 3-O—xylopyranosyl-(12)-O—galactopyranoside, Blumenol C malonylglycosyl galacturonide [M+H]+, Verbascoside. This study's phytochemical profiling of *T. cordifolia* indicated a wide variety of bioactive phenolics. *T. cordifolia* potent antidiabetic action can also be projected to be *T. cordifolia* phytochemical profiling was presented.

Bajpai *et al*. (2016) developed and validated LC-MS methods for the identification and simultaneous quantification of diverse secondary metabolites in the stems of male and female plants, as well as to investigate metabolomic differences in the stems of male and female plants, For fast screening of bioactive phytochemicals, an ethanolic extract of stems was analysed using HPLC/ESI-QTOF-MS/MS. Secondary metabolites were investigated structurally using high-resolution MS and MS/MS in positive ESI mode. For the simultaneous quantification of five bioactive alkaloids, a UPLC/ESI-QqQ (LIT)-MS/MS technique in MRM mode was designed and validated. LC-MS and MS/MS techniques were used to identify and characterise 36 metabolites, including alkaloids, sesquiterpenes, and phytoecdysteroids. Male and female plants were successfully quantified for bioactive alkaloids such as jatrorrhizine, magnoflorine, isocorydine, palmatine, and tetrahydropalmatine. Male plants had considerably greater mean abundances of magnoflorine, jatrorrhizine, and oblongine, whereas female plants had significantly higher mean abundances of tetrahydropalmatine, norcoclaurine, and reticuline. There were substantial qualitative and quantitative differences in phytochemicals in the stems of male and female *Tinospora cordifolia*. Based on their chemical profiles and amounts of the marker bioactive alkaloids, LC-MS and MS/MS technologies can be used to distinguish between male and female plants. This variation in chemical makeup was also visible during the vegetative stage, when there were no male or female flowers.

Deshmuk (2016) studied the isolation of bioactive compounds in plant *Tinospora cordifolia* and their biological role in disease cure and they reported the presence of different bioactive molecules like berbrine, choline, palmatine, tinosporin in the plant which is responsible for the eradication of disease.

Mohan *et al*. (2017) UPLC MS/MS Q-tof approach was developed to identify and characterise berberine in *Tinospora cordifolia* (Willd.) Miers. as well as to assess the bioactive fraction's anti-inflammatory potential. The results of HPLC and HPTLC investigations revealed the presence of berberine in *Tinospora cordifolia* methanolic extract, which was validated by

UPLC MS/MS Q-tof. In comparison to NDGA (positive control), which had an IC50 of 2.75 0.05 g/mL, the fraction containing berberine inhibited 5-LOX with an IC50 of 0.041 0.0003 g/mL. When berberine was docked with 5-LOX, it had a binding energy of -8.942 0.039665 kcal/mol and a Ki of 273.16 x 3.026 nM, whereas NDGA had a binding energy of -7.186 0.170503 kcal/mol and a Ki of 5.604 x 1.618 MT. *Tinospora cordifolia* can be utilised as a source of berberine, and the presence of berberine may be responsible for the plant's anti-inflammatory properties.

Saha and Bhakat (2017) reviewed that the wide range of therapeutic indications of *Tinospora cordifolia* (Guduchi) make it extreme popularity in Ayurveda. It has numerous synonyms having definite role to specify tridosha. Shaligram Nighantu classically mentioned the therapeutic indication of guduchi in jwara, daha, trishna, vaman, raktavikar, vatavyadhi, prameha, pandu and bhrama. Most specific indication are given by PN e.g., Jwara, prameha, kamala, vatrakta. Visharpa added by Shankar Nighantu which indicate that guruchi can use in emergency purpose.

Sultana *et al.* (2017) extracted air dried stem powder of *Tinospora cordifolia* with methanol. The concentrated methanolic extract was adsorbed on silica gel (60-120 mesh) for the preparation of a slurry. The dried slurry was chromatographed over silica gel column packed in petroleum ether. The elution was done with petroleum ether, chloroform and methanol successively in order of increasing polarity to isolate a variety of phytoconstituents including acetyl alcohol along with new chemical constituents characterized as trans-cinnamoyl-2-n-hexanyl-7-methoxynaphthyl amide, trans-cinnamoyl-2-n-pentanyl-6, 7-dimethoxynaphthyl amide, trans-cinnamoyl-2-n-octanyl-7-methoxynaphthyl amide, and 5-hydroxy-4'-methoxy-7-flavanoxy-(7→7")- β-O-labdan-1-en-3"α, 19"-olide-18"-oic acid (tinolabdenyl flavanone). They prepared the structures of all isolated phytoconstituents on the basis of spectral data analysis and chemical reactions.

Garg and Garg (2018) revealed the presence of flavonoids component in ethanol, methanol, chloroform solvent extracts of *Tinospora cordifolia*. Total Flavonoid Content (TFC) was high in methanolic and ethanolic leaves extract of *Tinospora cordifolia*. These results of plant sources were found to be highly significant.

Begum *et al.* (2019) quantified the major secondary metabolites and antioxidant potential of ethanolic leaf extract of *Tinospora cordifolia*. The ethanolic leaf extract of *Tinospora cordifolia* was analyzed by HPLC and GC to determine various phytochemicals. The ethanolic

extract of *Tinospora cordifolia* was found to contain alkaloids, terpenoids, phenols and flavonoids. The major flavonoid detected was quercetin and rutin. The *Tinospora cordifolia cordifolia* was found to possess significant radical scavenging activity against DPPH, NO and superoxide anions and the IC_{50} value of 42.0 µg/ml, 42.0 µg/ml and 42.6 µg/ml was observed. The medicinal property of *Tinospora cordifolia* may be attributed to the presence of flavonoids and phenolic compounds with rich antioxidant potential. The therapeutic effect of this plant may be accounted for its counteracting action on free radicals *in vivo.*

Taechowisan (2019) investigated major constituents of *Tinospora cordifolia* Miers. growing on *Mangifera indica* and evaluated the efficacy of their antibacterial and cytotoxicity activites. The ethanolic stem extract of *Tinospora cordifolia* was subjected to silica gel 60 column chromatography, thin layer chromatography and medium pressure liquid chromatography for isolation of the major compounds. Identification of purified compounds was achieved by spectroscopic methods. The crude extract and purified compounds were screened for their antibacterial and cytotoxicity properties using standard procedures. Two alkaloids were purified and identified as Magnoflorin and Tembetarine. These compounds showed high antibacterial activity against *Bacillus cereus* and *Staphylococcus aureus* with both MIC (Minimum Inhibition Concentration) (32-64 µg/ ml) and MBC (Minimum Bactericidal Concentration) (128-256 µg/ml). The cytotoxicity activity of the purified compounds and crude extract was determined using (3-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide) MTT colorimetric assay against L929 and HEK293 cell lines. This showed weak cytotoxicity activity with IC50 values of 1162.24 to 2290.00 µg/ml and 1376.67 to 2585.06 µg/ml towards L929 and HEK293 cell lines, respectively. This study suggests that these compounds exhibit great potential for antibacterial activity with weak cytotoxicity activity. They may be useful for their medicinal functions.

Krupanidhi *et al.* (2020) screened phytochemical compounds of *Tinospora cordifolia* for their inhibitory activity on SARS-CoV-2. On the basis of virtual screening and molecular docking analysis the phytochemical compounds, namely tinosponone, xanosporic acid, cardiofolioside B, tembetarine and berberine of *T. cordifolia* were identified as lead molecules to fight against SARS-CoV-2. The present *in silico* study proved that tinosponone as potent, selective and nontoxic inhibitor of 3CL protease of SARS-CoV-2.

Ahmad *et al.* (2020) concluded that Gilo is one of the best herbal plant which is used in (Unani System of Medicine) USM and also other system as an anticancer, blood purifier, antidiabetic, anti-inflammatory, antipyretic, antibacterial, immune modulation, hepatoprotective, aphrodisiac, snake-bite and carminative properties. The present review highlights only some of

its medicinal properties. Further research is needed, so that new herbal formulations can be prepared from the bioactive compounds of this important medicinal plant for the treatment of many fatal diseases.

Kumar *et al*. (2020) reviewed that all the parts of the plant *Tinospora cordifolia* are immensely useful due to the presence of different compounds of pharmaceutical importance belonging to various groups as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, and phenolics. These compounds possess pharmacological properties, which make it anti-microbial, anti-diabetic, anti-pyretic, anti-inflammatory, anti-oxidant, hepato-protective, and immuno-modulatory and revealed that there is need to be focus on phytochemistry, ethnopharmacology, clinical application and its conservation strategies so that the plant can be conserved for future generations and utilized as alternative medicine as well as to design various pharmacologically important drugs.

Saha and Ghosh (2020) studied the active components and biological role of *Tinospora cordifolia* and reported that the compounds like alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds, and polysaccharides have different biological role in disease conditions thus enable potential applications in clinical research. *Tinospora cordifolia* extracts are extensively used in various herbal preparations for the treatment of different ailments for its anti-periodic, anti-spasmodic, anti-microbial, anti-osteoporotic, anti-inflammatory, anti-arthritic, anti-allergic, and anti-diabetic properties. In case of anti-microbial activity, the methanol extracts of *Tinospora cordifolia* have been reported to have potential against microbial infections.

Srivastava and Singh (2021) reported that *Tinospora cordifolia* has been listed as an important plant amongst the 32 prioritized plants by National Medicinal Plants Board (NMPB), New Delhi of Government of India. *Tinospora cordifolia* has been used in Indigenous Systems of Medicine, as indicated in various classical texts of Ayurvedic System of Medicine, *viz.*, Charak, Sushrut and Ashtang Hridaya and other ancient treaties. It also finds a special mention for its use in tribal or folk medicine in different parts of the country. *Tinospora cordifolia* is an endangered rasayana herb of India and holds a special position as a potent adaptogen and aphrodisiac in Ayurvedic System of Medicine. The plant is rich in many phyto-constituents that are useful in drug designing.

II) Evaluation of antimicrobial potential of *Tinospora cordifolia*

Jeyachandran *et al.* (2003) studied the antibacterial activity of the aqueous, ethanol and chloroform extracts from the stems of *Tinospora cordifolia* by using disc diffusion method against *Escherichia coli, Proteus vulgaris, Enterobacter faecalis, Salmonella typhi* (Gram-negative), *Staphylococcus aureus and Serratia marcesenses* (Gram-positive) and revealed that the ethanolic extracts exhibited significant antibacterial activity against *Proteus vulgaris, Escherichia coli* and moderate activity was observed against *Enterobacter faecalis*. In the same extract less inhibition was observed against *Salmonella typhi, Staphylococus aureus and Serratia marcescens.* Hence, it is suggested that the ethanolic extract has significant antibacterial activity against tested bacteria.

Uddin *et al*. (2011) studied the antimicrobial and cytotoxic activities of *Tinospora cordifolia* by doing isolation of its secondary metabolites and evaluation of biological activity with different extract like methanol, chloroform, carbon tetrachloride and indicated that carbon tetrachloride and chloroform fractions were highly toxic.

Ilaiyaraja and Khanum (2011) reported the antioxidant potential of *Tinospora cordifolia* extracts and their protective effect on oxidation of biomolecules and their result demonstrate the potential of antioxidant activities of guduchi leafs as well as stem and therefore, it can be used as a source of antioxidant for health benefits through dietary supplementations.

Nagaprashanthi *et al*. (2012) evaluated the *in vitro* antifungal and antibacterial activity of hydro alcoholic extract of *Tinospora cordifolia* creeped on *Azadirachta indica* Tree (TC1) in comparison with that of *Tinospora cordifolia* (TC2) creeped on fencing. Hydroalcoholic extract of *T. cordifolia* stem was prepared by maceration technique. Various different microorganisms used as antibacterial and antifungal were *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Pseudomonas* spp.*, Aspergillus niger, Aspergillus fumigates, mucor* spp. *and Pencillium*. The extract of TC1 exhibit effective antimicrobial activity against all the organisms, while the extract of TC2 exhibits inhibition zone on limited species such like *Staphylococcus aureus* (12 mm), *Klebsiella pneumonia* (10 mm), *Pseudomonas* spp. (8 mm), *Aspergillus niger* (6 mm), *Aspergillus fumigates* (8 mm) and *Mucor* spp. (12 mm). They suggested that *T. cordifolia* creped on neem tree having the potential antimicrobial activity similar to *Azadirachta indica*. This explained that the host plants (*T. cordifolia*) will encorporate the medicinal virtue when they survive on neem plants.

Mishra *et al*. (2013) reported the scientific validation of *Tinospora cordifolia* for its medicinal efficacy which includes phytochemical screening, antimicrobial, antioxidant and anticancer activities of the plant. In disc diffusion assays, extracts (petroleum ether, chloroform, ethyl acetate, acetone, ethyl alcohol and water) exhibited considerable inhibition against *Klebsiella pneumoniae*. Several other extracts also showed antibacterial activity against pathogenic strains of *E. coli, Pseudomonas* spp., *and Proteus* spp. Minimum bactericidal concentration (MBC) values of potential extracts were found between 1.29 and 22.73 mg/mL. The lowest MBC (1.29 mg/mL) was recorded for acetone and ethyl acetate extracts against *K. pneumoniae* and *Pseudomonas* spp., respectively. The results established remarkable antibacterial, antioxidant, and anticancer potential in *Tinospora cordifolia* stem extracts.

Vermani *et al*. (2013) investigated the ability of *Tinospora cordifolia* crude extracts to inhibit the growth of dental (bacterial) pathogens *i.e., Staphylococcus aureus* (MTCC 1144), *Streptococcus mutans* (MTCC 890), *Streptococcus salivarius* (MTCC 1938), *Lactobacillus acidophilus* (MTCC 447), *Streptococcus sanguinis* (ATCC 10556) and their isolates. They observed that MeOH extract of *T. cordifolia* was most effective against all tested bacterial pathogens. Maximum antibacterial activity was observed against *S. sanguinis* (23 mm) and lowest activity against *S. salivarius* (17 mm). The phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins, phenols, saponins, glycosides, amino acids and steroids which might be accountable for its antimicrobial potential.

Reddy and Reddy (2015) summarized the anti-bacterial activity of *Tinospora cordifolia* extracts against *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhi, Shigella flexneri, Salmonella paratyphi, Salmonella typhimurium, Pseudomonas aeruginosa, Enterobacter aerogene,* and *Serratia marcesenses* (Gram-positive bacteria). Aqueous, ethanol and acetone extract of leaves and stem of *Tinospora cordifolia* Miers showed maximum inhibitory activity against of urinary pathogens *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Silver nanoparticles synthesized from stem of *Tinospora cordifolia* possess very good antibacterial activity against multi drug resistant strains of *Pseudomonas aeruginosa* isolated from burn patients. The active compound [(5R, 10R)-4R, 8R-Dihydroxy-2S, 3R:15, 16- diepoxycleroda13(16), 17, 12S, 18, 1S-dilactone] was isolated from ethanol extract of *Tinospora cordifolia* stem showed activity against bacteria and fungi. The lowest MIC values were observed against *Enterococcus faecalis* (125 µg/ml) and *Bacillus subtilis* (200 µg/ml). The compound also showed activity against fungi; the lowest minimum inhibitory concentration values were seen against *Trichophyton simii* (31.25 µg/ml), *Trichophyton rubrum* 57 (62.5 µg/ml*), Trichophyton rubrum* 296 (62.5 µg/ml).

George *et al*. (2016) reviewed the morphological, phytochemical and pharmacology aspects of *Tinospora cordifolia* (guduchi). All the parts of this plant are reported for various ethnobotanical and therapeutic uses. It is prescribed for many diseases such as general debility, fever, diabetes, dyspepsia, urinary infections, jaundice and skin diseases. Phytochemically, the various extracts showed the presence of diverse phytochemicals such as alkaloids, glycosides, polyphenols, steroids, tannins. Leaf of *Tinospora cordifolia* showed the maximum concentration of sugar, starch, flavonoids, phenolic, and tannin content as compare to aerial roots and stem. They also spotlight the classical antidiabetic, anticancer, immunomodulatory, antioxidant, antimicrobial, antitoxic claims of *Tinospora cordifolia* and their validation by contemporary researches.

Kaur *et al*. (2016) screened the phytochemical and biological aspect of *Tinospora cordifolia.* In Preliminary phytochemical screening of *T. cordifolia* they showed the presence of carbohydrates, glycosides, flavonoids, phenols, tannins and amino acids in the crude drug. They evaluate the antimicrobial activity of *T. cordifolia* against Gram +ve bacteria (*Staphylococcus aureus*) and Gram -ve bacteria (*Escherichia coli*) using the agar wells dilution method and showed that the stem extracts of *Tinospora cordifolia* exhibited marked dose dependent antimicrobial activity against both gram positive and gram-negative bacteria. Methanolic extract was found to be more potent against both the group of bacteria.

Singh and Saxena (2016) reported the screening of antimicrobial activity of *Tinospora cordifolia* and *Hymenocallis littoralis,* medicinal plants extracts with various different plant parts such as bulb, root, leaves and stem against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium* and fungi *Candida albicans* using agar well diffusion method. Antimicrobial efficiency of *Tinospora cordifolia* and *Hymenocallis littoralis,* medicinal plants were examined using ethanol, methanol, chloroform, dichloromethane, ethyl acetate as solvents and found that all the plants parts showed significant activity against all pathogens. The best MIC (Minimum Inhibitory Concentration) (zone of inhibition in mm) results were found in case of *Staphylococcus aureus* by using methanol stem crude extract of *Tinospora cordifolia.* In case of *Hymenocallis littoralis* the best MIC (zone of inhibition in mm) results were found in case *Escherichia coli* by using methanol bulb crude extract. The Spectrum of activity observed in the study may be indicative of that methanol extracts of these plants could be a possible source to obtain new and effective herbal medicines to treat different diseases or disorders.

Mamta and Jakhar (2016) investigated the *in vitro* antibacterial activity of *Tinospora cordifolia* stem extract against *Escherichia coli*. In serial two-fold dilution of *Tinospora cordifolia* stem aqueous extract, 1x107 colony forming units (CFU) of *Escherichia coli* were added. The turbidity corresponding to the bacterial growth in different dilutions was measured as optical density at 600 nm with a spectrophotometer. The maximum activity of extract was observed at its 1:32 dilution and minimum inhibitory concentration was found at its 1:64 dilutions. All the dilutions showed significantly lower CFU as compared to control positive. It can be concluded that *Tinospora cordifolia* stem extract possessed antibacterial activity against *Escherichia coli.*

Kumar *et al*. (2017) studied antimicrobial property of the leaves of amruthaballi. The leaves were tested and observed against microorganism *Escherichia coli*. Three different solvent extract *i.e.,* ethanolic, methanolic, and aqueous extracts of leaf were taken by using slip disc method, the leaf were tested for their degree of antimicrobial nature against *Escherichia coli*. They observed that the natural medicinal character of the climber is an economical alternative form of medicine compared to the currently used ones, with fewer side effects to the consumers and easy availability.

Salkar *et al.* (2017) describes the role of *Tinospora cordifolia* as an antimicrobial agent and as an immunity enhancer plant and their studies showed that *Tinospora cordifolia* possesses potential antimicrobial activity against uropathogens and can be used in UTI infections.

Patil *et al*. (2017) studied the antibacterial and phytochemical analysis of *Tinospora cordifolia, Azarchita indica* and *Ocimum sanctum* leaves extract against common human pathogens like *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Enterobacter aerogenes, Pseudomonas aeruginosa, Salmonella typhimurium, Salmonella typhi, Staphylococcus epidermidis* and *Proteus vulgaris.* They revealed the presence of anthraquinones, alkaloids, saponins, tannins, glycosides and phenolics and also suggested that the identified phytochemicals may be the bioactive constituents responsible for the efficacy of leaves extract of *Tinospora cordifolia, Azarchita indica* and *Ocimum sanctum* against fever, syphilitic, ulcer, inflammatory disease wounds, conjunctivitis, etc.

Prajwala *et al.* (2018) demonstrated the potent role of *Tinospora cordifolia* antibacterial property against *Escherichia coli* cell division. *Tinospora cordifolia* leaf extract was used as test compound to inhibit the growth of *Escherichia coli* with Ampicillin as reference. Methanolic

extract showed zone of inhibition or inhibitory effect on growth of *Escherichia coli*. Compared to the remaining extracts which may be attributed to chemical constituents responsible for antibacterial activity are more soluble in methanolic extract.

Sharma *et al.* (2019) examined the antimicrobial, antioxidant, qualitative phytochemicals screening and FT-IR analysis of some folk medicinal plants (*Viola odorata* flower, *Tinospora cordifolia* stem, *Bacopa monnieri* leaves and *Mentha piperita* leaves) and they concluded that all the selected folk medicinal plants can be used as alternative herbal treatment of diseases. *Viola odorata* and *Tinospora cordifolia* had antioxidant properties. Aqueous extract of both plants had the presence of various phytochemicals that can be used as pharmaceutical products.

Prasad and Chauhan (2019) evaluated the antimicrobial property of ethanolic and methanolic extract of root and stem of *Tinospora cordifolia* against some pathogenic microorganisms viz., *Escherichia coli, Bacillus subtilis, Aspergillus niger and Candida* spp. It was found that the various concentration of extract *viz*., 50, 100, 150 and 200 mg ml^{-1} were tested and was observed that the increase in concentration also increased antimicrobial activity, revealed by increase in size of zone of inhibition. The methanolic stem extract exhibits highest antimicrobial activity against all four pathogens. The study showed that extract of *Tinospora cordifolia* has a wide range of anti-oxidant as well as antimicrobial activity against bacterial as well as fungal pathogens.

Priti and Rani (2019) studied comparative estimation of antimicrobial and antioxidant activity of leaves and stems of *Tinospora cordifolia* and revealed that the stem part of *Tinospora cordifolia* contains higher quantity of phytochemicals, due to which it showed better antibacterial and antioxidant activity than leaf tissue and thus has higher prospective applications in food, agriculture and pharmaceutical industry.

Pratihast *et al.* (2019) evaluated and compared the effects of ethanolic (EtOH) and aqueous (H_2O) extracts of *Tinospora cordifolia* (Menispermaceae) plant extract. Phytochemical screening of the plant extract showed the presence of different percentage of active phytochemicals such as alkaloid, flavonoid, phenolic, saponin, tannin etc. The ethanolic extract and aqueous extracts in agar diffusion test at the concentrations of 250 mg/ml and 125 mg/ml also showed an effectual zone of inhibition against all tested micro-organisms.

Sharma *et al.* (2019) reviewed the diverse pharmalogical importance of *Tinospora cordifolia viz*., antifungal activity, antioxidant activity, antimicrobial activity, antibacterial activity, hepatic disorder, anticancer, anti-HIV potential, antitoxic effects, immunomodulating

activity, systemic infection, and Parkinson's disease. It has been used successfully in Ayurvedic medicine from the ancient era, and its products are used for their better economic and therapeutic utilization.

Prajwala *et al*. (2019) studied the *in vitro* anti-bacterial activity of *Tinospora cordifolia* leaf extract against *E. coli* by using disc diffusion method. They revealed that among aqueous, methanolic, chloroform, hexane and acetone extract only methanolic extract showed zone of inbhibition against *E. coli*. In antibacterial activity methanolic extract was effective against *E. coli.*

Agarwal *et al.* (2019) tested antimicrobial activity of different concentrations of *Tinospora cordifolia* against *S. mutans.* Seven different concentrations of *in vitro Tinospora cordifolia* obtained by 100 per cent agar medium. Plates were incubated aerobically at 37^0C for 48 hours and zone of inhibition was measured. 0.2 per cent chlorhexidine and dimethylforamide were used as +ve and -ve control respectively. Maximum antibacterial activity was observed with zone of inhibition of 19mm chlorohexidine showed zone of inhibition of 28mm at 2 per cent concentration, whereas, no zone of inhibition was observed with dimethyformamide. Hence, they concluded that *Tinospora cordifolia* exhibited antimicrobial activity against *S. mutans.*

Wai *et al.* (2019) reported qualitative phytochemical screening of the stems of *Tinospora cordifolia* Miers. by using standard methods. The stems of *Tinospora cordifolia* Miers. revealed the presence of alkaloids, flavonoids, terpenoids, phenolic compounds, saponins, tannins, glycosides and carbohydrates, and showed the absence of steroids and reducing sugars. The quantitative crude alkaloid was extracted from the stems of *Tinospora cordifolia* Miers. by using Harbone J.B. Method. The percentage yield of the crude alkaloid was found to be 2.008 per cent. And the antimicrobial activities of crude alkaloid extracted from the stems of *Tinospora cordifolia* Miers. showed high activities on all tested organisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albicans* and *Escherichia coli*. The stems of *Tinospora cordifolia* Miers. could serve as a potential source of useful drugs.

Barua *et al.* (2020) investigated the antimicrobial property from the extract of *Tinospora cordifolia* stem by using agar diffusion method against some gram-negative *Salmonella paratyphi, Salmonella tiphi, Vivrio parahemolyticus, Vivrio mimicus, Escherichia coli, Shigella dysenteriae, Pseudomonas aureus,* and *Shigella boydii* and gram-positive bacteria - *Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus, Sarcina lutea*. They observed the zone of

inhibition formed by the methanolic extract in the antimicrobial assays and concluded that the methanolic extract of *Tinospora cordifolia* may possess significant antimicrobial property which requires more studies to isolate the specific bioactive compound for developing a new antimicrobial agent from this plant.

Gnanam *et al*. (2020) evaluated the antibacterial activity of methanolic stem fraction of *Tinospora cordifolia* against *Escherichia coli* and *Staphylococcus aureus* and in result they showed the maximum inhibition of the growth of bacteria were ranged from 2 mm to 6 mm for *E. coli* and 1.5 mm to 6.3±0.29 mm for *S. aureus*. They also concluded that methanolic stem fraction of *T. cordifolia* possesses promising therapeutic activity against the urinary tract infection pathogens such as *E. coli* and *S. aureus*. Further exploration in isolation and characterization such as plant-derived phytoconstituents would open up new ventures in the field of antibacterial drug discovery.

Arshan *et al.* (2020) evaluated the antimicrobial activity of medicinal plants such *as Asparagus racemosus, Cyperus rotundus, Zingiber officinale, Terminalia bellirica, Tinospora cordifolia, Acacia concinna* and *Cedrus deodara* against different Gram-positive and Gram-negative bacterial strains. The evaluation of antimicrobial activity of each plant extracts was carried out using the disc diffusion method. The antibacterial activity of aqueous extracts *of Terminalia chebula, Justicia adhatoda, Terminalia bellirica, Withania somnifera* and *Zingiber officinale* were found to be equally effective compared to standard antibiotics. *Terminalia chebula* and *Terminalia bellirica* showed maximum activity against selected fungal strains. The result confirmed the effectiveness of certain selected plant extracts as natural antimicrobials and suggested the possibility of using them in drugs for the treatment of infectious diseases caused by test organisms.

Bhat *et al*. (2020) analyzed that *Tinospora cordifolia* is used as a immunomodulating herb. One of compounds, 1,4-alpha-D-glucan derived from *Tinospora cordifolia* has been found to activate macrophages, NFKB translocation and cytokine production, and hence activates the immune system. In present time no specific treatment or vaccine for novel coronavirus -2019 is there. From the past experiences we have learnt that *Tinospora cordifolia* have proven beneficial against various dreadful viral infections. So, after assessment of immune enhancing power of *Tinospora cordifolia* it is definitely helpful for the body to fight with COVID-19 infections.

Upadhyay *et al*., (2021) analyzed the antioxidant activity of leaf and stem extracts of *Tinospora cordifolia* by using DPPH (1,1-Diphenyl-2-picrylhydrazyl) and ABTS (2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) free radical scavenging assays. The result showed that

the Methanolic stem extract showed higher phenolic and flavonoid content along with antioxidant activity as compared to the methanolic leaf extract. And concluded that the stem extract exhibited more antioxidant activity than the leaf extract with regards to the all parameters analyzed.

Chapter - 3

Materials and Method

The present investigations on the "Quantification of bioactive molecules and evaluation of antimicrobial potential of *Tinospora cordifolia* Miers." was carried out in the Department of Biotechnology, College of Horticulture and Forestry (Dr. Y.S. Parmar University of Horticulture and Forestry), Neri, Hamirpur (HP) during 2020-2021. The outlines of methodology followed to carry out the above-mentioned investigations have been described under following captions: -

3.1 Quantification of bio active molecules of *Tinospora cordifolia*

3.1.1 Plant materials

3.1.1.1 Source of plant materials

3.1.1.2 Glassware and instruments

3.1.2 Authentication

3.1.3 Drying

3.1.4 Grinding

3.1.5 Sample preparations

3.1.5.1 Preparation of methanolic extract

3.1.5.2 Preparation of ethanolic extract

3.1.5.3 Preparation of chloroform extract

3.1.5.4 Preparation of aqueous extract

3.1.6 High performance thin layer chromatography analysis (HPTLC)

3.1.6.1 Optimization of solvents

3.1.6.2 Procedure of HPTLC

3.1.7 Liquid chromatography mass spectrophotometry

3.1.7.1 Sample preparation for LCMS

3.1.7.2 Procedure for LCMS

3.1.7.3 Mass spectrometry parameters

3.1.7.4 Data processing using mass hunter software

3.2 Evaluation of anti-microbial potential of *Tinospora cordifolia.*

3.2.1 Plant materials

3.2.2 Preparation of extract

3.2.2.1 Preparation of methanolic extract

3.2.2.2 Preparation of ethanolic extract

3.2.2.3 Preparation of chloroform extract

3.2.2.4 Preparation of aqueous extract

3.2.3 Collection of microbial samples

3.2.4 Inoculum preparation

3.2.5 Media preparation and its sterilization

3.2.6 Preparation of plates

3.2.7 Antimicrobial activity

3.2.8 Minimum inhibitory concentration and Minimum bactericidal concentration

3.2.9 Statical analysis

3.1 Quantification of bio active molecules of *Tinospora cordifolia*

3.1.1 Plant material

3.1.1.1 Source of plant material

The plant material of *Tinospora cordifolia* was collected from the different regions of Kangra, Hamirpur and Mandi districts of Himachal Pradesh for isolation of compounds. The number and name of villages is given below:

Table No. 3.1: Location of sample collecting sites

S. No.	Name of the district	No. of villages	Name of village	Plant part used
01	Hamirpur	02	Kotlangsan Neri	Roots, Stem and leaves

02	Kangra	02	Samloti Palampur	Roots, Stem and leaves
03	Mandi	02	Jhodan Manwana	Roots, Stem and leaves

3.1.1.2 Glassware and instruments

During experiment the forceps, scalpel handle, scalpel blade, pH meter, weighing balance, micropipette, autoclave, laminar air flow, hot air oven, refrigerator and gas was used. Some culture vessels and culture apparatus also used like beaker, petri plate Kolkata, measuring cylinder, test tubes, erlenmeyer flask and cotton plugs etc.

3.1.2 Authentication

Collected plant samples are identified by the Department of Forest Products, College of Horticulture and Forestry, Neri, Hamirpur (H.P.) for future studies.

3.1.3 Drying

After the collection of the samples, it needs to be dried to make the sample extract. In general, the plant material should be dried to avoid the decomposition of thermolabile compounds. Plant drying under the sun are very effective, but the disadvantage is sometimes water molecules are soaked by the sample, and hence microbial growth can affect the phytochemical study. All the sample are dried at room temperature under shadow, grounded and stored at the room temperature till further use.

3.1.4 Grinding

A small quantity of plant material is milled using grinder or blender. But if the sample is in excessive quantity, then it is less complicated to get a powdered sample by means of grinding from a spice mill. Grinding improves the performance of extraction through the increased surface area. It also decreases the quantity of solvent required for the extraction. The dried samples were ground into a coarse powder with the help of a mechanical grinder (Blender) and powdered samples had been kept in clean closed glass packing containers pending extraction. At the time of grinding of the sample, the grinder cleaned to keep away from contamination with any remnant of earlier ground material.

3.1.5 Sample preparations

The powdered plant materials are dissolved by the usage of specific solvents *viz.*, Ethanol, Methanol, Chloroform and water via maceration approach to gain the crude extracts. The method used for sample preparation was done in with the accordance of Taechowisan (2019) with some modifications.

3.1.5.1 Preparation of methanolic extract

The plant samples (root, stem and leaves) powder was thoroughly washed with water, cut into small pieces and dried in hot air oven. After drying, samples were coarsely powdered using a suitable grinding mill. About 2.5 g of powdered material was macerated with methanol at room temperature for a period of 2 days followed by occasional shaking and stirring. After that the plant extract was filtered with clean cotton filter followed by whatman filter paper No. 1. The solvent was evaporated on water bath at 40 ºC. After complete drying the extract is scraped from the mortar. The extract was then preserved in a refrigerator (2-4 ºC) till further use.

3.1.5.2 Preparation of ethanolic extract

The plant samples (root, stem and leaves) powder was thoroughly washed with water, cut into small pieces and dried in hot air oven. After drying samples were then coarsely powdered using a suitable grinding mill. About 2.5 g of powdered material was macerated with ethanol at room temperature for a period of 2 days with occasional shaking and stirring. After that the plant extract was filtered with clean cotton filter followed by whatman filter paper No. 1. The solvent was evaporated on water bath at 40 ºC. After complete drying the extract is scraped from the mortar. The extract was then preserved in a refrigerator (2-4 ºC) till further use.

3.1.5.3 Preparation of chloroform extract

The plant samples (root, stem, leaves) powder was thoroughly washed with water, cut into small pieces and dried in hot air oven. After drying samples were then coarsely powdered using a suitable grinding mill. About 2.5 g of powdered material was macerated with chloroform at room temperature for a period of 2 days followed by occasional shaking and stirring. After that the plant extract was filtered with clean cotton filter followed by whatman filter paper No. 1. The solvent was evaporated on water bath at 40 ºC. After complete drying the extract is scraped from the mortar. The extract was then preserved in a refrigerator (2-4 ºC) till further use.

3.1.5.4 Preparation of aqueous extract

The plant samples (root, stem and leaves) powder was thoroughly washed with water, cut into small pieces and dried in hot air oven. After drying samples were then coarsely powdered using a suitable grinding mill. About 2.5 g of powdered material was macerated with water at room temperature for a period of 2 days followed by occasional shaking and stirring. After that the plant extract was filtered with clean cotton filter followed by whatman filter paper No. 1. The solvent was evaporated on water bath at 40 ºC. After complete drying the extract is scraped from the mortar. The extract was then preserved in a refrigerator (2-4 ºC) till further use.

3.1.6 High performance thin layer chromatography (HPTLC) analysis

3.1.6.1 Optimization of solvents

In order to optimize the best solvent for extraction for HPTLC, various combination of solvents *i.e.,* Methanol (100%), Ethanol (100%), EM (50:50), EM (80:20), Water, Chloroform were checked and the solvents with good results was used further (Bala *et al.* 2015).

3.1.6.2 Procedure for HPTLC

- HPTLC was performed on 20×20 cm normal phase TLC aluminium phase pre coated with 200 mm layer thickness of silica gel $60F_{254.}$
- Analytical HPTLC was carried out according to the modified method of (Pesci *et al*. 1999).
- For analytical HPTLC, firstly fresh sample 1mg/ml in the suitable solvent was prepared from the previous stored samples.
- Marked the line above the lower edge using pencil and name of the sample was spotted on the plate.
- 10 µL of each prepared sample was applied on the TLC plate leaving at least 1 cm distance between the samples.
- TLC chamber was prepared with best solvent and kept for 15 min for solvent system to equilibrate.

- Plate was placed in TLC chamber and the solvent was allowed to migrate until the 80 % of plate is covered or minimum 45 h.
- Plate was removed from TLC chamber, air dried and visualized under UV light to observe the fractions and bands.
- RF value were calculated by using formula.

$$\mathrm{RF} = \frac{Distance\ covered\ by\ solute}{Distance\ covered\ by\ solvent}$$

After HPTLC get all the RF values and the sample containing highest RF value further used for doing LC-MS.

3.1.7 LC-MS (liquid chromatography – mass spectrophotometer)

3.1.7.1 Sample preparations

For LC-MS prepared a fresh sample which get the highest RF value. 5 g of powdered material was macerated with suitable solvent at room temperature for a period of 5 days followed by occasional shaking and stirring. After that the plant extract was filtered with clean cotton filter followed by Whatman filter paper (No. 1). The solvent was evaporated on water bath at 40 ºC. After complete drying the extract is scraped from the mortar. The extract was then preserved in a refrigerator (2-4 ºC) till further use. The sample preparation for LC-MS was done accordance to Ahmed *et al.* (2006) with some modification.

3.1.7.2 Procedure of liquid chromatography-mass spectrometry (LC-MS)

A gradient elution was used for separation using Agilent 6560 LC-MS. An acquity BEH C18 column (2.1 mm x 100 mm, 1.7 µm) was used. Mobile phase A contained 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile in B. The gradient started from 5 % B at 0 min, further increased from 5-15 % till 5 min, then 15-25 % B for 5-6 min, 40 % B from 6-12 min, 50 % B from 12-14 min, 58 % B from 14-20 min. The concentration of mobile phase then decreased to 25 % from 20-22 min and mobile phase maintained to initial conditions 5 % B at 25 min. Elution was performed at a solvent flow rate of 0.2 mL/min.

3.1.7.3 Mass spectrometry parameters

Mass spectrometry parameters were: ionization mode, ESI positive; drying gas temperature, 300 °C; drying gas flow, 5.0 L/min; nebulizer gas pressure, 35 psi; capillary voltage, 3500 V; fragmentor voltage, 400 V; detection window, 100 ppm; MS scan range and rate, 100-3000 m/z at 1 spectra/s; MS threshold, 200; MS/MS threshold, 5.

3.1.7.4 Data processing using mass hunter software

Peaks were extracted from the total ion chromatograms (TICs) using Agilent's Mass Hunter Qualitative Analysis software (v. B.06.00, Agilent technology).

3.2 Evaluation of anti-microbial potential of *Tinospora cordifolia*.

3.2.1 Plant material

The plant material of *Tinospora cordifolia* was collected from the different regions of Kangra, Hamirpur and Mandi districts of Himachal Pradesh for isolation of compounds. The number and name of villages are given below:

Table No. 3.2 : Locations of sample collecting sites

S. No.	Name of the district	No. of villages	Name of village	Plant part
01	Hamirpur	02	Kotlangsan Neri	Roots, Stem and leaves
02	Kangra	02	Samloti Palampur	Roots, Stem and leaves
03	Mandi	02	Jhodan Manwana	Roots, Stem and leaves

From the collected samples roots, stems and leaves were washed, dried and crushed to coarse powder for preparation of extract. The preparation of extract was done with the accordance of Singh *et al*. (2016), Saxena *et al*. (2020) by doing slightly modification.

3.2.2 Preparation of Extract

3.2.2.1 Preparation of methanolic extract

3 g powdered *Tinospora cordifolia* (root, stem, and leaves) was macerated with methanol at room temperature for 6 days with occasional shaking and stirring and then filtered. The extract was then filtered by using whattman filter paper no. 1 and evaporated using water bath. The residue obtained after evaporation was stored in sterile eppendorf vails for further use.

3.2.2.2 Preparation of ethanolic extract

3 g powdered *Tinospora cordifolia* (root, stem, and leaves) was macerated with ethanol at room temperature for 6 days with occasional shaking and stirring and then filtered. The extract was then filtered by using whattman filter paper no. 1 and evaporated using water bath. The residue obtained after evaporation was stored in sterile eppendorf vails for further use.

3.2.2.3 Preparation of chloroform extract

3 g powdered *Tinospora cordifolia* (root, stem, and leaves) was macerated with chloroform at room temperature for 6 days with occasional shaking and stirring and then filtered. The extract was then filtered by using whattman filter paper no. 1 and evaporated using water bath. The residue obtained after evaporation was stored in sterile eppendorf vails for further use.

3.2.2.4 Preparation of aqueous extract

3 g powdered *Tinospora cordifolia* (root, stem, and leaves) was macerated with water at room temperature for 6 days with occasional shaking and stirring and then filtered. The extract was then filtered by using whattman filter paper no. 1 and evaporated using water bath. The residue obtained after evaporation was stored in sterile Eppendorf vails for further use.

3.2.3 Collection of microbial sample

The cultures *viz*., of *Staphylococcus aureus* (MTCC 1144), *E. coli* (MTCC-1667), *Pseudomonas aeruginosa* (PA01) and *Aspergillus niger* (MTC-282) were kindly provided by Dr. Geeta Shukla Department of Microbiology and Biotechnology, Punjab University, Chandigarh.

3.2.4 Inoculum preparation

S. aureus, E. coli, P aeruginosa and *A. niger* used as test organisms. Cultures of bacteria were grown for 12 to 24 hours in nutrient broth and LB Broth at 37 ºC. Culture of Fungus were grown for 24 to 48 hours in on potato dextrose agar at 30 ºC.

3.2.5 Media preparation and its sterilization

For disk diffusion method (Bauer *et al.* 1996), antimicrobial susceptibility was tested on solid (Agar-agar) media in petri plates. For bacterial assay nutrient agar (NA) (40 gm/L) and for fungus PDA (39 gm/l) was used for developing colonies on the surface of the medium. The minimum inhibitory concentration (MIC), was determined by Serial tube dilution technique. The suspension culture, for bacterial cells growth was done by preparing 2 % Lauria Broth (w/v), and for fungal growth 2.4 % (w/v) PDB (Potato dextrose broth) was taken for evaluation. All media prepared were then sterilized by autoclaving at (121°C) for 20 min.

3.2.6 Preparation of plates

Prepared agar media sterilized and cooled till 50 °C in a water-bath. Pouring of about 20 ml agar into pre-labelled sterile Petri dishes. They were then permitted to set at room temperature and were dried so that no drops of moisture remained on the surface of the agar.

3.2.7 Antimicrobial activity

Antibacterial activity of *Tinospora cordifolia* leaf extract was carried out using disc diffusion method (Perez *et al*. 1990) against *S. aureus, E. coli, P. aeruginosa* and *A. niger* with aqueous, ethanolic, methanolic and chloroform extract were used as test compound and gentamycin is used as reference standard. Briefly10 µl of bacteria (10^6cells/CFU/ml) was inoculated and streaked on agar plate using cotton swabs. Disc containing different concentration of *Tinospora cordifolia* leaf stem and root extract were plates inoculated. Plates were incubated overnight for 37 ºC for 24 h of *S. aureus, E. coli, P. aeruginosa* and 28º C for 48 h of *A. niger*. Zone of inhibition was measured after 24 h and 48 h. The zone of inhibition was measured and the extract having maximum antibacterial activity was used for further use.

3.2.8 Minimum inhibitory concentration and minimum bactericidal concentration

- MIC of plant extract was determined against *P. aeruginosa* (PA01), *S. aureus* by standard microbroth dilution method according to National Committee for Clinical Laboratory Standards.
- MIC of plant extract was evaluated against *P. aeruginosa* (PA01), *S. aureus* by the calorimetric method using Microtiter Plate Assay described by Webster *et al*. (2010).
- Standard strain *P. aeruginosa* and *S. aureus* was cultivated in LB media under shaking conditions for 6 h to obtain a cell count of 10^6 cells/ml.
- 200µL of LB with inoculum (*P. aeruginosa* and *S. aureus*) was added to each well of a sterile 96 well microtiter plate.
- Stock solution of plant extract of different concentration in normal saline were prepared.
- 100 µL each of tested plant extract was added to the first well of each column and then it was serially diluted from first well to sixth well in each row.
- The last column containing 100 µL LB media served as negative control.
- The plate was incubated at 37 ºC for 16-18 h. The maximum dilution with no visible growth was taken as MIC.

Subramanian *et al.* (2002), Doughari *et al.* (2008) reported the procedure for the determination of MIC and MIC the same pattern was followed wit.

3.2.9 Statistical analysis

Experiments were set up in a completely randomized block (CRD) design (Cochran and Cox, 1963 and Gomez and Gomez, 1984) and each experiment had three replicates. The results were expressed as mean ± SE of the experiments. The data were analysed using two-way (ANOVA). The statistical analysis was carried out by using MS-Excel and OPSTAT.

Chapter - 4

Result and Discussion

Tinospora cordifolia is an important drug of Indian systems of medicine and used in medicines since times immemorial. The drug is well known Indian bitter and prescribed in fevers, diabetes, dyspepsia, jaundice, urinary problems, skin diseases and chronic diarrhoea and dysentery. It has been also indicated useful in the treatment of heart disease, leprosy, and helmenthiasis. A variety of active components derived from the plant like alkaloids, steroids, diterpenoid lactones, aliphatic, and glycosides have been isolated from the different parts of the plant body, including root, stem, and whole plant (Upadhyay *et al*. 2010). In the recent times the plant has gained a lot of attention of the researchers because of its pharmaceutical importance and medicinal properties such as anti-cancer, anti-spasmodic, anti-inflammatory, anti-oxidant, anti-malarial, immunoprotective, immunomodulatory and anti- tuberculosis activities (Ansari *et al.* 2016; Shanthi and Nelson, 2013).

The present research entitled "Quantification of bioactive molecules and evaluation of antimicrobial potential of *Tinospora cordifolia* Miers" was carried out to standardize the protocol for HPTLC and LC-MS for quantification of bioactive molecules and checking the antimicrobial potential of plants from different locations.

4.1 Sample collection

The medicinal properties and biological functions of bioactive compounds of the *Tinospora cordifolia* in the control of disease have led to the interest in the plant and therefore the plant sample were collected from 03 different districts from Himachal Pradesh. Roots, stems and leaves of *Tinospora cordifolia* were collected from 06 different locations of Himachal Pradesh in the months of July, August in the year of 2020. Plant sample were collected from two villages of districts Hamirpur *i.e.,* Kotlangsan and Neri, two villages of districts Mandi *i.e.,* Jodan and Manwana and two villages of districts Kangra *i.e.,* Samloti and Maniyara.

Table 4.1 Locations of sample collection sites

S.No.	Name of the district	No. of villages	Name of village	Plant part
01	Hamirpur	02	Kotlangsan Neri	Roots, Stem and leaves
02	Kangra	02	Samloti Manyiara	Roots, Stem and leaves
03	Mandi	02	Jhodan Manwana	Roots, Stem and leaves

4.2 High performance thin layer chromatography (HPTLC) analysis.

4.2.1 Comparison of extraction solvent.

In order to optimize the best solvent for obtaining more number of fractions and highest RF value the various solvents were used *i.e.,* ethanol (100%), methanol (100%) and ethanol: methanol (50:50) were applied in the same solid to solvent ratio. The comparison of these solvents are in followings tables.

Table 4.2 Calculation of RF values of samples of *Tinospora cordifolia* in (Methanol 100%)

S.No	Locations	Samples	Distance travelled by solvent (cm)	No. of fractions	Distance travelled by fractions of solute (cm)	RF
1	Hamirpur (Kotlangsan)	RM	8.9	1	1.2	0.13
2		SM	8.9	1	4.2	0.47
3		LM	8.9	3	7.6 8.2 8.3	0.85 0.92 0.93
4	Hamirpur (Neri)	RM	8.9	1	1.2	0.13
5		SM	8.9	1	3.9	0.43
6		LM	8.9	3	7.6 8.3 8.4	0.85 0.93 0.94

The HPTLC analysis of *Tinospora cordifolia* was done with methanol (100%). The result indicated the highest RF value of 0.94 in sample collected from village Neri of district Hamirpur.

Table 4.3 Calculation of RF values of samples of *Tinospora cordifolia* in (Ethanol100%)

S.No	Location	Samples	Distance travelled by solvent (cm)	No. of fractions	Distance travelled by fractions of solute (cm)	RF
1	Hamirpur (Kotlangsan)	RM	9.2	01	1.1	0.11
2		SM	9.2	02	1.1 8.5	0.11 0.92
3		LM	9.2	03	4.9 5.9 6.6	0.53 0.64 0.71
4	Hamirpur (Neri)	RM	9.2	02	0.4 8.7	0.04 0.94
5		SM	9.2	01	8.9	0.96
6		LM	9.2	02	5.8 6.3	0.63 0.68

The HPTLC analysis of *Tinospora cordifolia* was done with ethanol (100%). The result indicated the highest RF value of 0.94 in sample collected from village Neri of district Hamirpur

Table 4.4 Calculation of RF values of samples of *Tinospora cordifolia* in (ethanol: methanol (50:50))

S.No	Locations	Samples	Distance travelled by solvent (cm)	No. of fractions	Distance travelled by fractions of solute (cm)	RF
1	Hamirpur (Kotlangsan)	KRM	9.1	03	1.1 7.8 8.8	0.12 0.85 0.96
2		KSM	9.1	02	8.1 8.6	0.89 0.94
3		KLM	9.1	01	8.4	0.92
4	Hamirpur (Neri)	NRM	9.1	04	0.5 7.3 7.9 8.9	0.05 0.80 0.86 0.97
5		NSM	9.1	03	6.6 7.5 8.4	0.72 0.82 0.92
6		NLM	9.1	04	6.5 7.1 7.9 8.5	0.71 0.78 0.86 0.93

The HPTLC analysis of *Tinospora cordifolia* was done with (ethanol: methanol (50:50)). The result indicated the highest RF value of 0.97 in sample collected from village Neri of district Hamirpur.

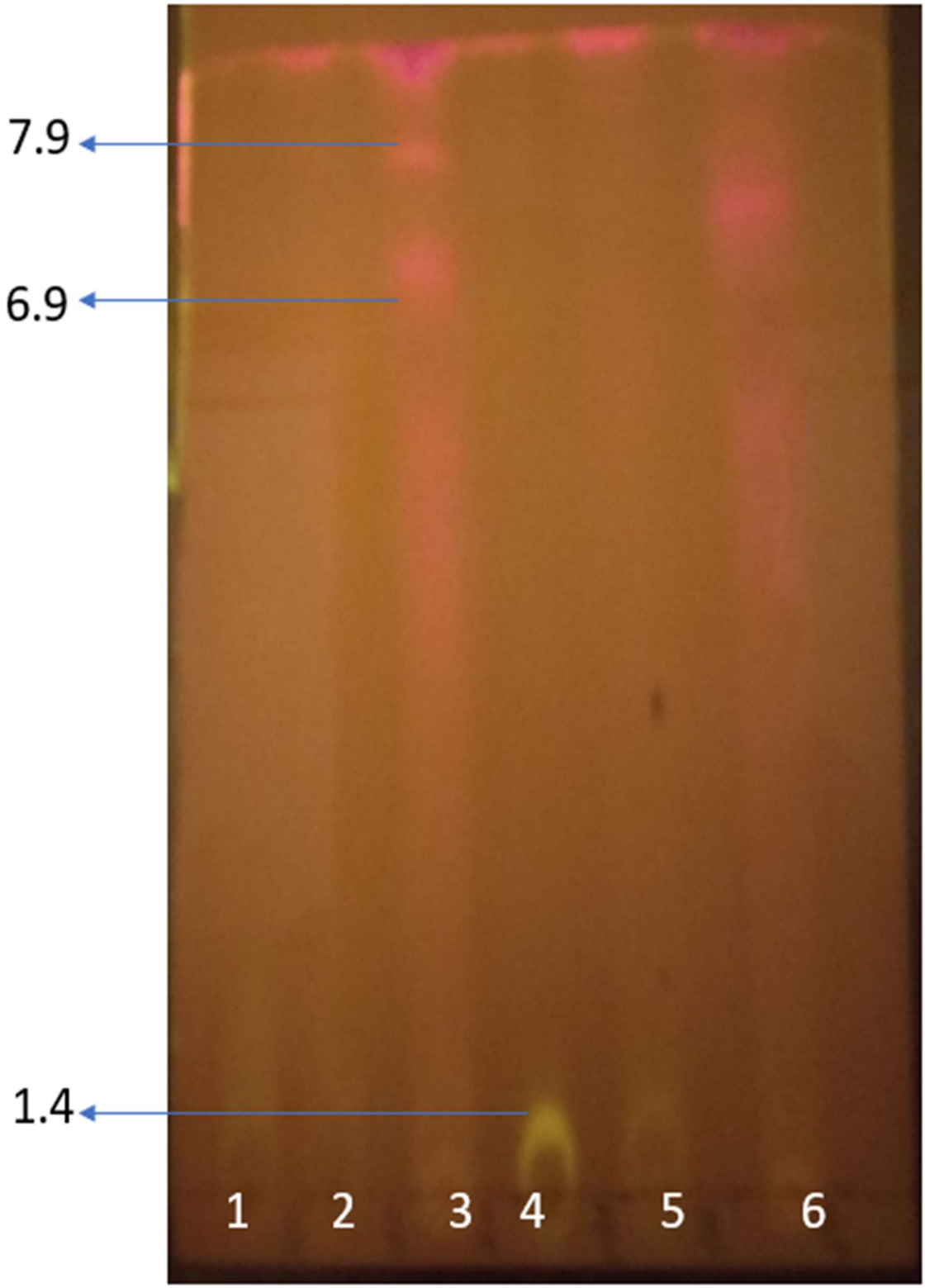
Among different solvents used the (ethanol: methanol (50:50)) was found to be the best for the *Tinospora cordifolia*. The result was indicated by the presence of highest RF value. Therefore (ethanol: methanol (50:50)) was used to calculate other RF values. Bala *et al.* (2015) reported the same solvent in order to optimize the best solvents for the extraction of bioactive compounds. They used various combination of solvent *i.e.,* M (100), E (100), M: W (50:50), E: W (50:50), E:M (50:50).

As depicted in plate 1 TLC plates *i.e.,* thin layer chromatography shows the separated bands which shows the presence of bioactive compounds in the running solvent ethanol: methanol in the ratio of 50:50. The number of fractions present in different extract varied from being maximum in methanolic extract followed by ethanol extract and chloroform extract whereas, minimum number of fragments were observed in case of aqueous extract as shown sample number C.

4.2.2 HPTLC of aqueous samples of all locations of *Tinospora cordifolia*

Table 4.5 Calculation of RF values of aqueous samples of all locations of *Tinospora cordifolia*

S.NO	Location	Samples	Distance travelled by solvent (cm)	No. of fractions	Distance travelled by fractions of solute (cm)	RF
1	Hamirpur (Kotlangsan)	RW	9.1	01	1.4	0.15
2		SW	9.1	02	1.4 6.5	0.15 0.71
3		LW	9.1	03	6.5 6.9 8.2	0.71 0.75 0.90
4	Hamirpur (Neri)	RW	9.1	01	7.0	0.76
5		SW	9.1	02	6.0 6.8	0.65 0.74
6		LW	9.1	01	7.6	0.83
7	Mandi (Jodan)	RW	9.1	01	6.5	0.71
8		SW	9.1	01	6.5	0.71
9		LW	9.1	02	6.5 7.5	0.71 0.81
10		RW	9.1	02	6.4 7.5	0.70 0.81
11	Mandi	SW	9.1	01	6.4	0.70



PLATES-1. On TLC plate separated bands showing the presence of bioactive compounds in solvents ethanol: methanol (50:50) in aqueous sample of root (1,4) , stem (2,5) and leaves (3,6) of *Tinospora cordifolia* Miers.

12	(Manwana)	LW	9.1	03	6.5 7.1 7.4	0.71 0.78 0.81
13	Kangra (Samloti)	RW	9.1	01	6.9	0.75
14		SW	9.1	01	7.1	0.78
15		LW	9.1	03	5.3 6.9 7.9	0.58 0.75 0.86
16	Kangra (Manyiara)	RW	9.1	01	1.9	0.20
17		SW	9.1	01	7.1	0.78
18		LW	9.1	02	5.9 6.7	0.64 0.73

From table 4.5 we have observed that, in location 1 (kotlangsan) of district Hamirpur the leaf aqueous extract had maximum number of fractions compared to aqueous root extract or aqueous stem extract. This was also confirmed by the highest RF value (0.91) in aqueous leaf extract. Similarly in second location (Neri) of district Hamirpur the leaf aqueous extract had the highest RF value 0.83 as compare to aqueous root extract or aqueous stem extract.

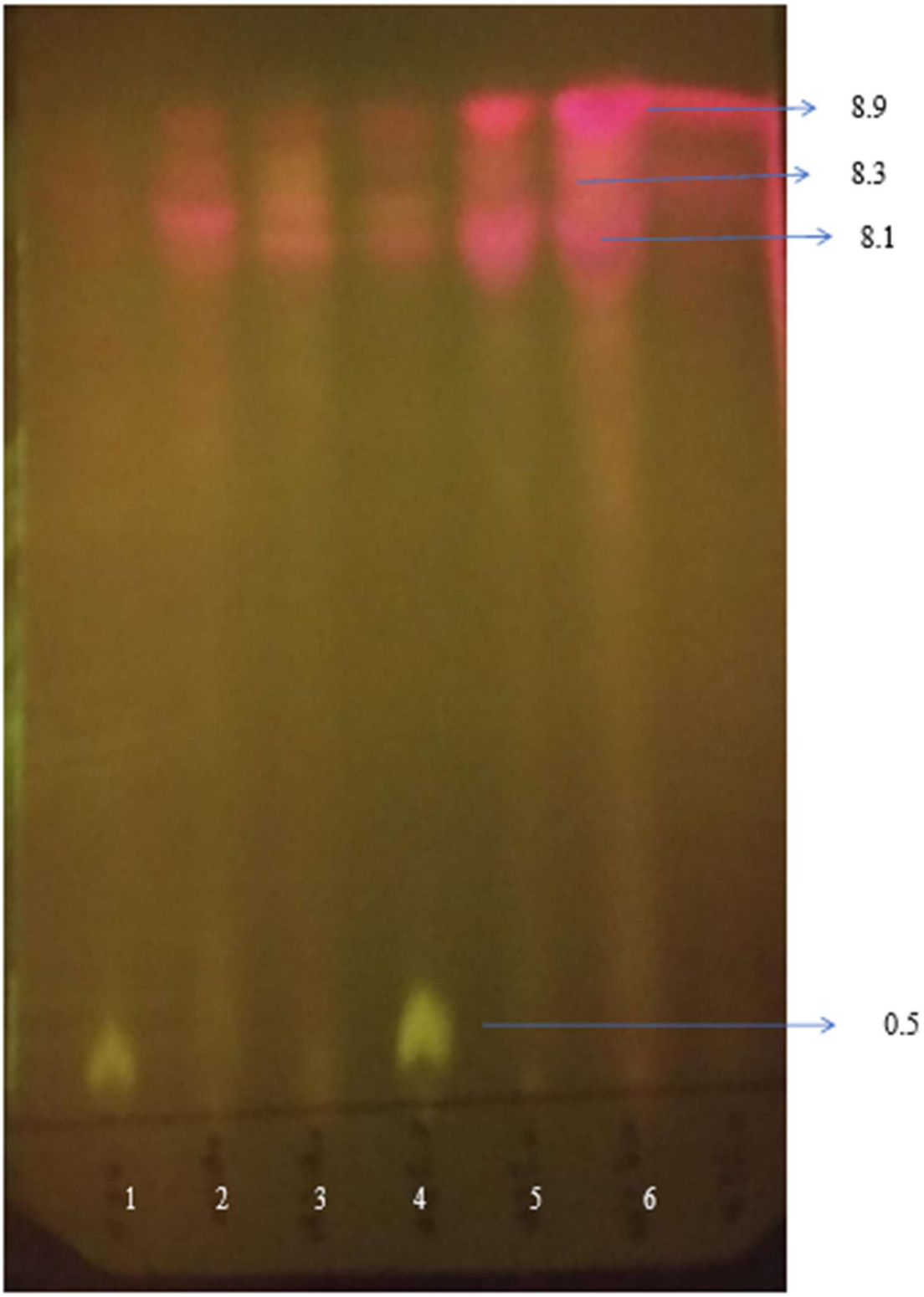
In location 1 (Jodan) of district Mandi the leaf aqueous extract had maximum number of fractions compared to aqueous root extract or aqueous stem extract. This was also confirmed by the highest RF value (0.81) in leaf aqueous extract. Similarly in second location (Manwana) of district Mandi had maximum number of fractions in the leaf aqueous extract had the highest RF value 0.81 as compare to aqueous root extract or aqueous stem extract.

In location 1 (Samloti) of district Kangra the leaf aqueous extract had maximum number of fractions compared to aqueous root extract or aqueous stem extract. This was also confirmed by the highest RF value (0.86) in leaf aqueous extract. Similarly in second location (Manyiara) of district Kangra had maximum number of fractions in the leaf aqueous extract had the highest RF value 0.73 as compare to aqueous root extract or aqueous stem extract.

From the above findings we have conclude that the leaf aqueous extract of all location has highest RF value and from these all the location 1 i.e., Kotlangsan of district Hamirpur had the highest RF value.

Table 4.6 Calculation of RF values of ethanol samples of all locations of *Tinospora cordifolia*

S.No	Locations	Samples	Distance travelled by solvent (cm)	No. of fractions	Distance travelled by fractions of solute (cm)	RF
1	Hamirpur (Kotlangsan)	RE	9.1	1	1.4	0.15
2		SE	9.1	1	4.4	0.48
3		LE	9.1	4	7.8 8.4 8.5 8.6	0.85 0.92 0.93 0.94
4	Hamirpur (Neri)	RE	9.1	1	1.4	0.15
5		SE	9.1	1	4.1	0.45
6		LE	9.1	4	7.8 8.5 8.6 8.7	0.85 0.93 0.94 0.95
7	Mandi (Jodan)	RE	9.1	03	0.6 7.6 7.8	0.06 0.83 0.85
8		SE	9.1	02	6.6 8.6	0.72 0.94
9		LE	9.1	05	0.4 6.5 7.4 8.1 8.6	0.04 0.71 0.81 0.89 0.94
10	Mandi (Manwana)	RE	9.1	03	0.5 7.9 8.1	0.05 0.86 0.89
11		SE	9.1	03	0.5 7.9 8.4	0.05 0.86 0.92
12		LE	9.1	01	7.8	0.85
13	Kangra (Samloti)	RE	9.1	03	8.1 8.3 8.4	0.89 0.91 0.92
14		SE	9.1	03	1.7 8.1 8.3	0.18 0.89 0.91
15		LE	9.1	03	7.2 8.1 8.3	0.79 0.89 0.91
16	Kangra (Manyiara)	RE	9.1	04	1.4 7.1 7.7 8.4	0.15 0.78 0.84 0.92
17		SE	9.1	02	1.2 8.2	0.13 0.90



PLATES-2. On TLC plate separated bands showing the presence of bioactive compounds in solvents ethanol: methanol (50:50) in ethanolic sample of root (1,4), stem (2,5) and leaves (3,6) of *Tinospora cordifolia* Miers.

18		PE	9.1	03	7.2 7.8 8.3	0.79 0.85 0.91

From table 4.6 we have observed that, In location 1 (Kotlangsan) of district Hamirpur the leaf ethanolic extract had maximum number of fractions compared to root ethanolic extract or stem ethanolic extract. This was also confirmed by the highest RF value 0.94 in leaf ethanolic extract. Similarly in second location (Neri) of district Hamirpur the leaf ethanolic extract had the maximum number of fraction and highest RF value 0.95 as compare to root ethanolic extract or stem ethanolic extract.

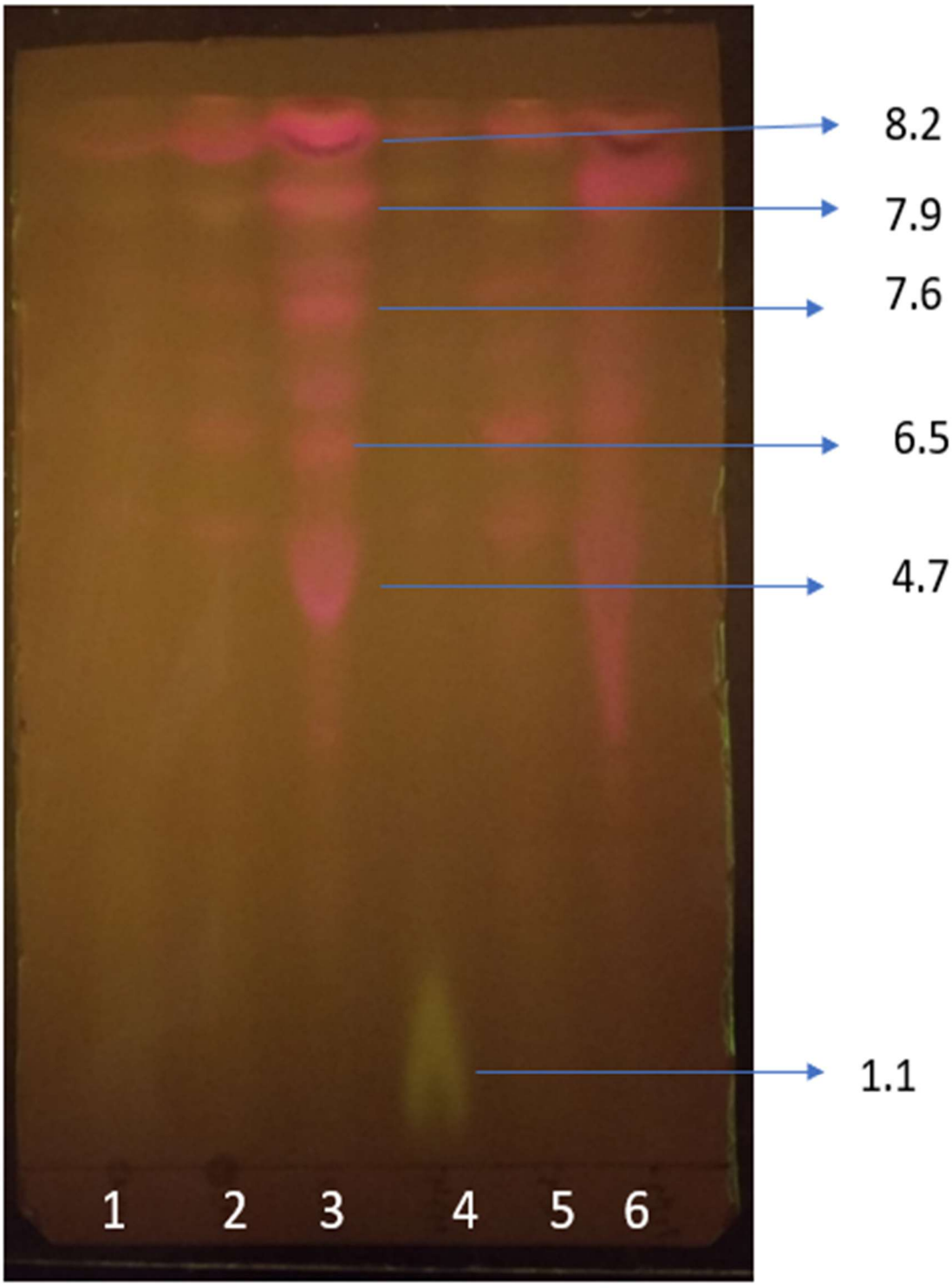
In location 1 (Jodan) of district Mandi the leaf ethanolic extract had maximum number of fractions compared to root ethanol extract or stem ethanol extract. This was also confirmed by the highest RF value 0.94 in leaf ethanol extract. In second location (Manwana) of district Mandi had maximum number of fractions in the stem ethanol extract had the highest RF value 0.92 as compare to root ethanol extract or stem ethanol extract.

In location 1 (Samloti) of district Kangra the root, stem, leaf ethanolic extract had equal number of fractions and root ethanolic extract has highest RF value (0.92). In second location (Manyiara) of district Kangra had maximum number of fractions in the root ethanol extract had the highest RF value 0.92 as compare to stem ethanol extract or leaf ethanol extract.

From the above findings we have conclude that the leaf ethanol extract of location 2 Neri district Hamirpur have highest RF value.

Table 4.7 Calculation of RF values of methanolic samples of all locations of *Tinospora cordifolia*

S.No	Locations	Samples	Distance travelled by solvent (cm)	No. of fractions	Distance travelled by fractions of solute (cm)	RF
1	Hamirpur (Kotlangsan)	RM	9.1	03	1.1 7.8 8.8	0.12 0.85 0.96
2		SM	9.1	02	8.1 8.9	0.89 0.97
3		LM	9.1	01	8.4	0.92
4	Hamirpur	RM	9.1	04	0.5	0.05



PLATES-3. On TLC plate separated bands showing the presence of bioactive compounds in solvents ethanol: methanol (50:50) in sample of root (1,4) stem (2,5) and leaves (3,6) of *Tinospora cordifolia* Miers.

	(Neri)				7.3 7.9 8.6	0.80 0.86 0.94
5		SM	9.1	03	6.6 7.5 8.4	0.72 0.82 0.92
6		LM	9.1	04	6.5 7.1 7.9 8.5	0.71 0.78 0.86 0.93
7	Mandi (Jodan)	RM	9.9	03	0.5 1.0 8.1	0.05 0.10 0.89
8		SM	9.9	03	7.3 7.9 8.2	0.80 0.86 0.89
9		LM	9.1	03	7.4 7.6 8.5	0.81 0.83 0.93
10	Mandi (Manwana)	RM	9.1	02	0.9 7.6	0.09 0.83
11		SM	9.1	03	7.4 7.9 8.2	0.81 0.86 0.89
12		LM	9.1	04	7.5 8.1 8.3 8.5	0.82 0.89 0.91 0.93
13	Kangra (Samloti)	RM	9.1	03	0.5 7.6 8.1	0.05 0.83 0.89
14		SM	9.1	03	0.4 7.6 8.2	0.04 0.83 0.90
15		LM	9.1	04	0.5 7.5 8.4 8.9	0.05 0.82 0.92 0.97
16	Kangra (Manyiara)	RM	9.1	03	7.3 7.9 8.4	0.80 0.86 0.92
17		SM	9.1	02	8.4 8.8	0.92 0.95
18		LM	9.1	05	4.7 6.5 7.6 7.9 8.2	0.51 0.71 0.83 0.86 0.90

From table 4.7 we have observed that, In location 1 (Kotlangsan) of district Hamirpur the root methanolic extract had maximum number of fractions compared to stem methanolic extract or leaf methanolic extract. This was also confirmed by the highest RF value 0.96 in root methanolic extract. Similarly in second location (Neri) of district Hamirpur the root methanolic extract and leaf methanolic extract had the maximum number of fraction and highest RF value 0.94 in root methanolic extract.

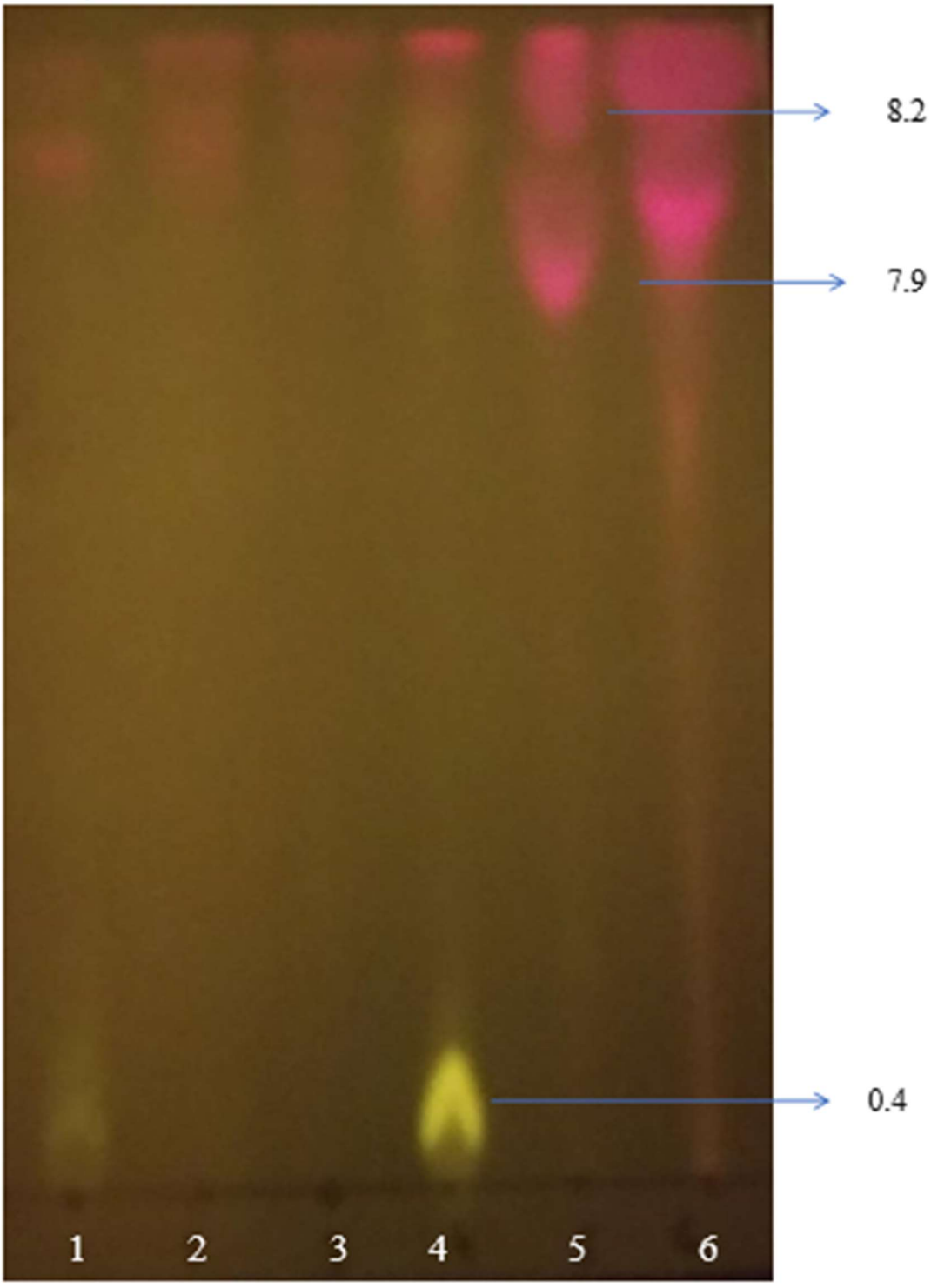
In location 1 (Jodan) of district Mandi the root, stem, leaf methanolic extract had same number of fractions. The highest RF value is 0.93 in leaf methanolic extract. In second location (Manwana) of district Mandi had maximum number of fractions in the leaf methanol extract and the highest RF value 0.93 as compare to root methanol extract or stem methanol extract.

In location 1 (Samloti) of district Kangra the leaf methanolic extract had maximum number of fractions and leaf methanolic extract has highest RF value 0.97. In second location (Manyiara) of district Kangra had maximum number of fractions in the leaf methanol extract had the highest RF value 0.90 as compared to stem ethanol extract or root methanol extract.

From the above findings we have conclude that the leaf methanol extract of location 1 Samloti district Kangra have highest RF value.

Table 4.8 Calculation of RF values of chloroform samples of all locations of *Tinospora cordifolia*

S.No	Locations	Samples	Distance travelled by solvent (cm)	No. of fractions	Distance travelled by fractions of solute (cm)	RF
1	Hamirpur (Kotlangsan)	RC	9.1	01	0.4	0.04
2		SC	9.1	01	7.4	0.81
3		LC	9.1	02	7.4 8.2	0.81 0.96
4	Hamirpur (Neri)	RC	9.1	03	0.3 7.3 8.2	0.03 0.80 0.90
5		SC	9.1	03	7.4 7.9 8.2	0.81 0.86 0.90
6		LC	9.1	01	7.5	0.82



PLATES-4. On TLC plate separated bands showing the presence of bioactive compounds in solvents ethanol: methanol (50:50) in chloroform extract of root (1,4) stem (2,5) and leaves (3,6) of *Tinospora cordifolia* Miers.

7	Mandi (Jodan)	RC	9.1	01	0.4	0.04
8		SC	9.1	02	8.3 8.9	0.91 0.93
9		LC	9.1	02	8.4 8.8	0.92 0.96
10	Mandi (Manwana)	RC	9.1	02	0.6 8.6	0.06 0.96
11		SC	9.1	02	8.4 8.7	0.92 0.95
12		LC	9.1	01	8.8	0.96
13	Kangra (Samloti)	RC	9.1	02	0.4 7.4	0.04 0.81
14		SC	9.1	02	7.4 8.3	0.81 0.91
15		LC	9.1	02	7.4 7.9	0.81 0.86
16	Kangra (Manyiara)	RC	9.1	01	7.6	0.83
17		SC	9.1	01	7.9	0.86
18		LC	9.1	01	7.9	0.86

From 4.8 we have observed that, In location 1 (Kotlangsan) of district Hamirpur the leaf chloroform extract had maximum number of fractions compared to root chloroform extract or stem chloroform extract. This was also confirmed by the highest RF value 0.96 in leaf chloroform extract. In second location (Neri) of district Hamirpur the root chloroform extract and stem chloroform extract had the maximum number of fraction and highest RF value 0.90 in root and stem chloroform extract.

In location 1 (Jodan) of district Mandi the stem and leaf chloroform extract had same number of fractions. The highest RF value is o.96 in leaf chloroform extract. In second location (Manwana) of district Mandi had maximum number of fractions in the root and stem chloroform extract and the highest RF value 0.96.

In location 1 (Samloti) of district Kangra the root, stem and leaf chloroform extract had maximum number of fractions and stem chloroform extract has highest RF value 0.91. In second location (Manyiara) of district Kangra had equal number of fractions in the root, stem and leaf chloroform extract and the highest RF value 0.86 in stem and leaf chloroform extract.

From the above findings we have conclude that the leaf chloroform extract of location 1*i.e.,* Kotlangsan district Hamirpur and location 3 Jodan district Mandi have highest RF value.

After comparing all the RF values of root, stem and leaves ethanolic, methanolic, chloroform and aqueous extracts in result we found that the leaves methanolic samples of *Tinospora cordifolia* get the highest RF value of district Kangra village Samloti and the value is 0.97. So, we done the LC-MS analysis of this sample.

Kaur *et al.* (2016) reported the phytochemical analysis of *Tinospora cordifolia* through thin layer chromatography and in methanolic extract the RF value are 0.05,0.1,0.3,0.55,0.72,0.76,0.83 and 0.87. Similarly in chloroform extract the RF value are 0.09, 0.69, 0.74, 0.78, 0.86. Albinjose *et al.* (2015) suggested the number of fractions and RF values of methanolic and chloroform extract of Tinospora cordifolia. The number of fractions in chloroform extract is 4 and 2 fractions had RF value is 0.15 and 0.18. the number of fractions in methanolic extract is 5 and three fraction had RF values is 0.67, 0.71 and 0.93.

4.2.3 LC – MS analysis

Table 4.9 Mass spectral characteristics of the 11 standard compounds of *Tinospora cordifolia* analysed by LC-MS.

S.no	Compounds	ESI-MS m/z [identity]
1	Magnoflorine	342
2	Menisperine	356
3	20b-Hydroxyecdysone	498,481,480,463,445,427
4	2-Deoxy-20b-hydroxyecdysone-3-O-glucopyranoside	609,627
5	Columbinyl glucoside	538
6	Columbamine	338
7	Jatrorrhizine	338
8	Palmatine	352

9	2-Deoxy-20b-hydroxyecdysone	482,465,464,447,429,411
10	Berbrine	336,338,339
11	Columbin	315,359,376

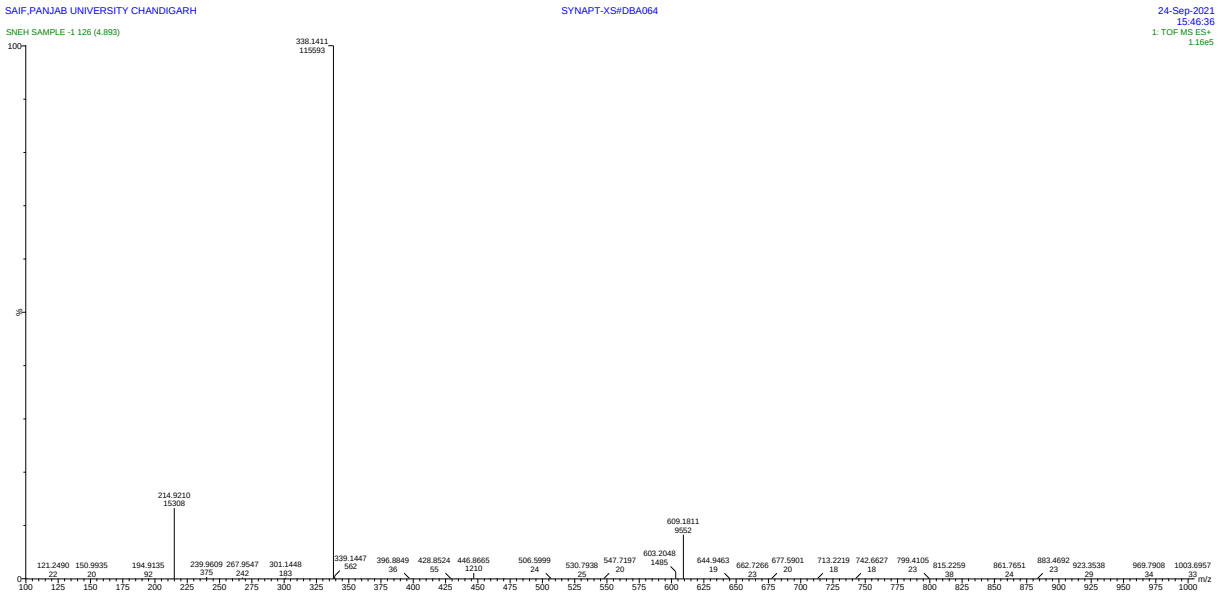


Fig. 4.1 Mass spectrometry pattern of leaf methanolic extract of *Tinospora cordifolia* showing the presence of Berbrine

It is evident from the figure 4.2 that the molar mass of berbrine was found to be 338.71g/mol which is very close to molar mass of standard berbrine *i.e.*, 336.1. the observation is similar to those observed by Mohan *et al.* (2017), Krupanidhi *et al.* (2020) who have calculated the m/z ratio of berbrine near to 336.3.

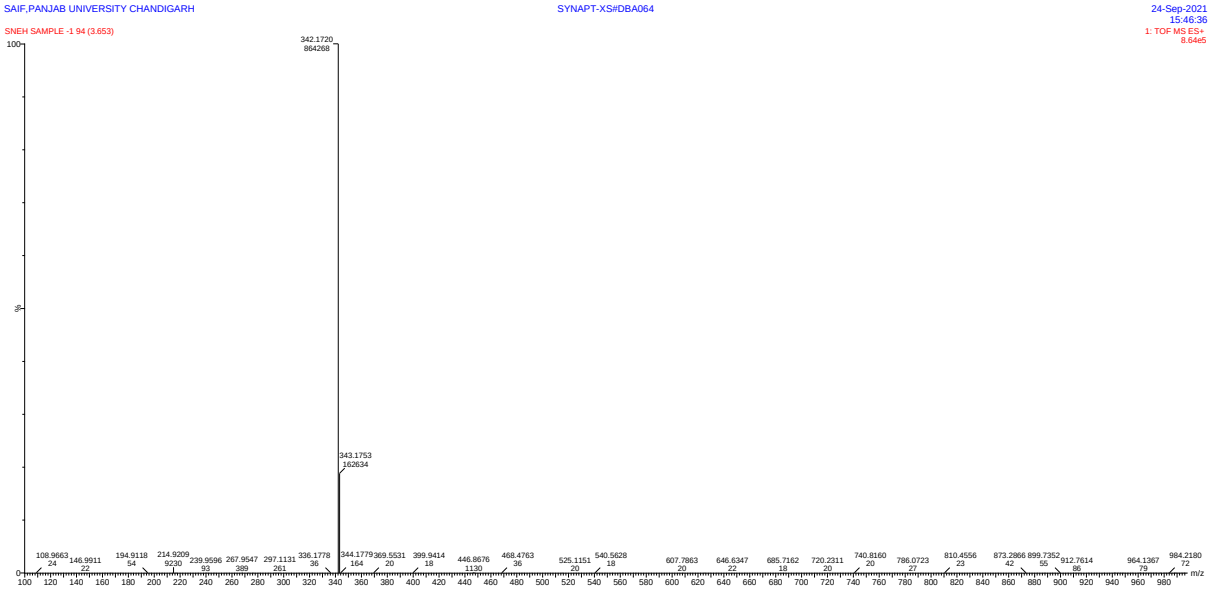


Fig. 4.2 Mass spectrometry pattern of leaf methanolic extract of *Tinospora cordifolia* showing the presence of magnoflorine

It has been observed that the sharp peak has obtained having molecular weight 342.17 g/mol corresponded to Magnofluorine. The present research has been validated by similar findings from Bajpai *et al.* (2015) who have reported that similar peak at 342 m/z ratio

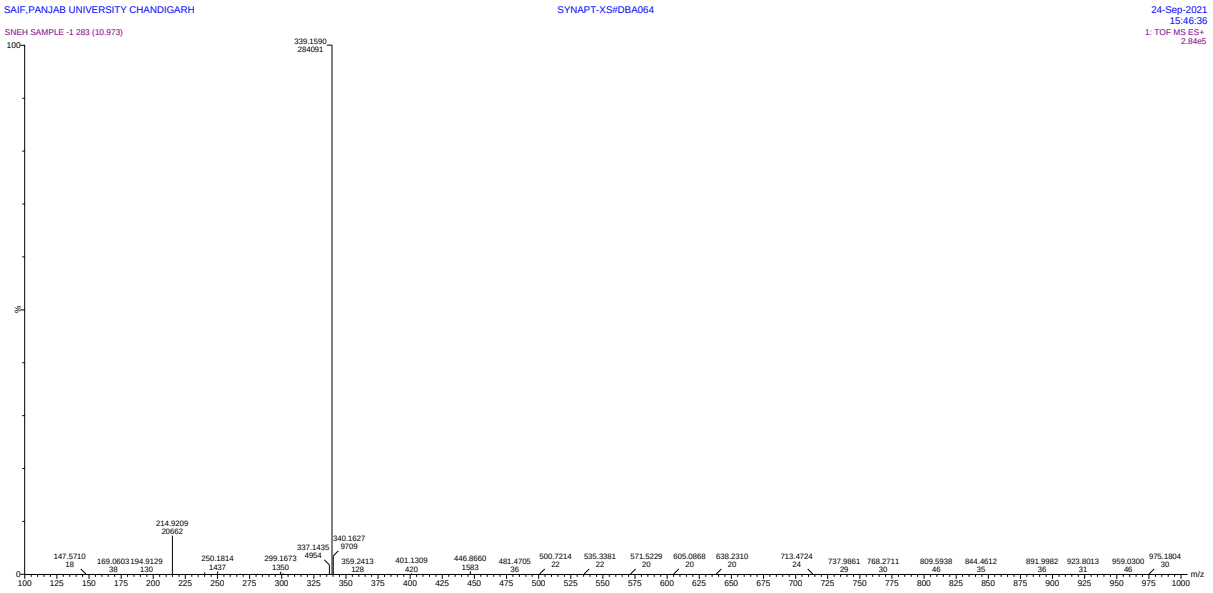


Fig. 4.3 Mass spectrometry pattern of leaf methanolic extract of *Tinospora cordifolia* showing the presence of columbaine.

It has been observed that the sharp peak has obtained having molecular weight 339.15 g/mol corresponded to Columbaine. Ahmed *et al*. (2006**)** reported the same compounds with 339.47g/mol on the basis of LC-MS.

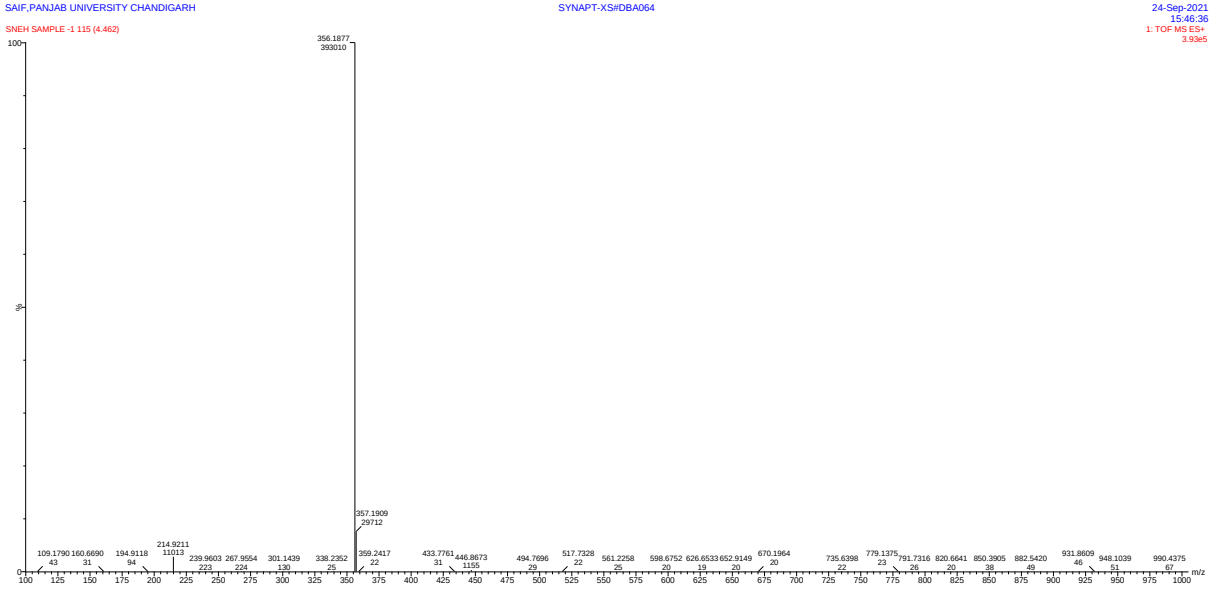


Fig. 4.4 Mass spectrometry pattern of leaf methanolic extract of *Tinospora cordifolia* showing the presence of menisperine

It is evident from the figure 5 that the molar mass of Menisperine was found to be 356.18g/mol which is very close to molar mass of standard berbrine *i.e.*, 356.1. the observation are very much similar to those observed by Zhang *et al.*, (2006) who have calculated the m/z ratio of menisperine near to 356.3

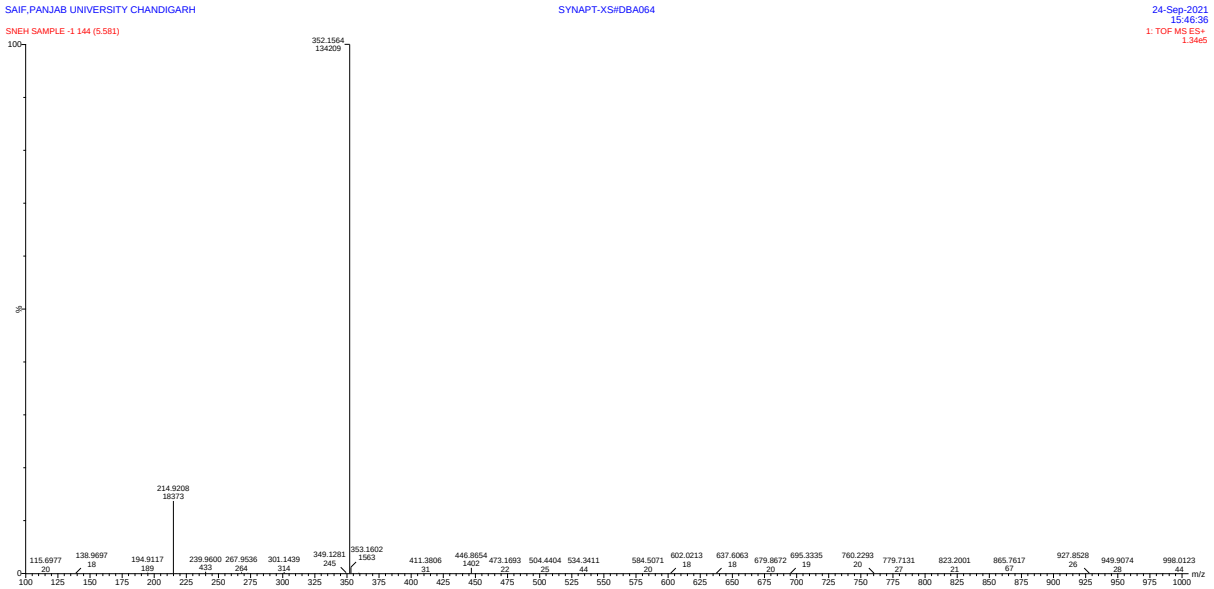


Fig. 4.5 Mass spectrometry pattern of leaf methanolic extract of *Tinospora cordifolia* showing the presence of palmatine

The above figure shows the sharpest peak with the molecular weight 352.15 g/mol which was similar to the molecular weight of palmatine *i.e.,* 352.15g/mol. Bajpai *et al.* (2015) showed the mass transition of palmatine between 352.1 to 336.0 which is similar to our observance.

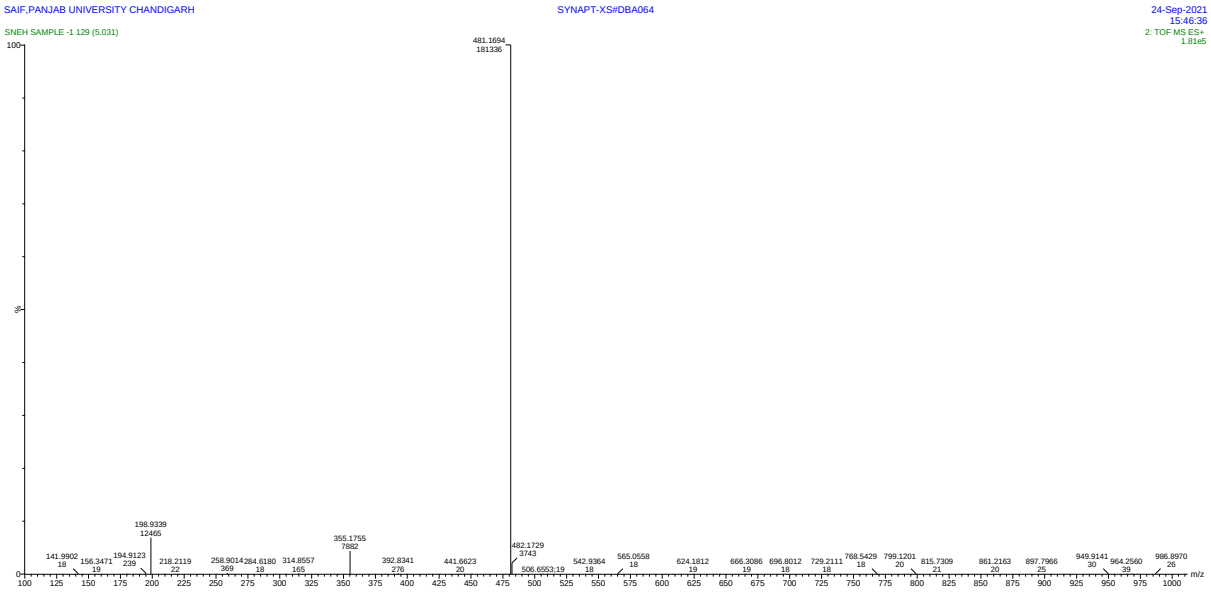


Fig. 4.6 Mass spectrometry pattern of leaf methanolic extract of *Tinospora cordifolia* showing the presence of 20b-Hydroxyecdysone

The above MS Spectra shows the sharpest peak with the molecular mass 481.16 g/mol which was similar to the molecular weight of 20b-hydroxyecdysone *i.e.,* 481.16g/mol. Zhang *et al. (*2006) showed the mass transition of 20b-hydroxyecdysone between 498.1 to427. 0 which is similar to our observance.

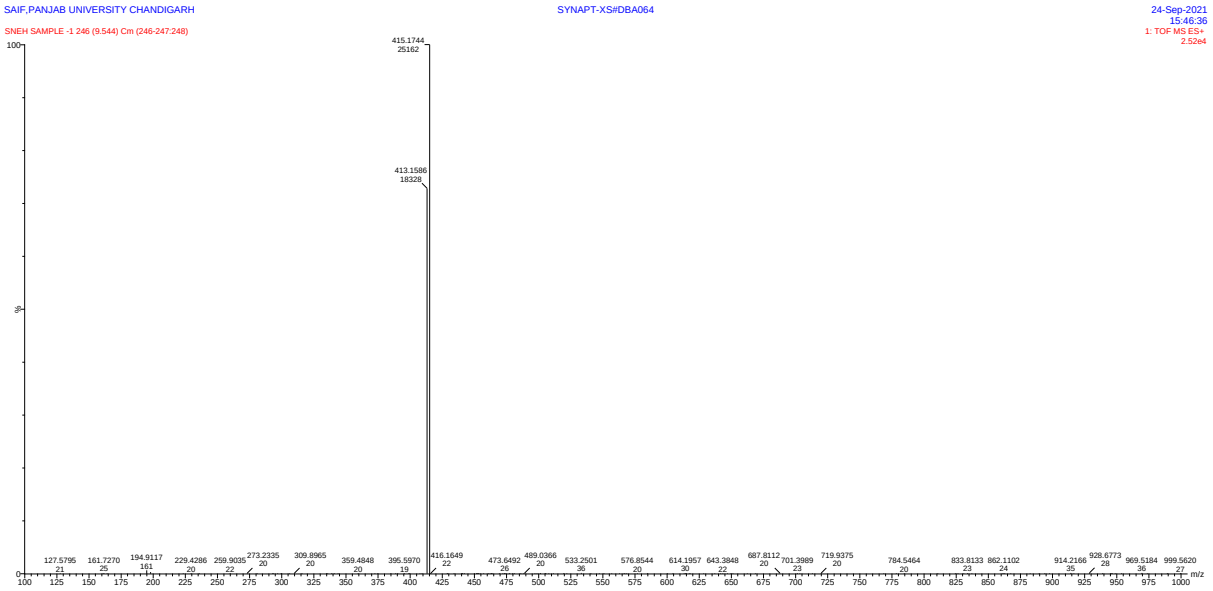


Fig. 4.7 Mass spectrometry pattern of leaf methanolic extract of *Tinospora cordifolia* showing the presence of 2-Deoxy-20b-hydroxyecdysone

The fig. 4.7 MS Spectra shows the sharpest peak with the molecular mass 415.17 g/mol which was similar to the molecular weight of 2-deoxy-20b-hydroxyecdysone i.e 415.16g/mol. Zhang *et al.* (*2006)* showed the mass transition of 2-deoxy-20b-hydroxyecdysone between 482 to 411which is similar to our observance.

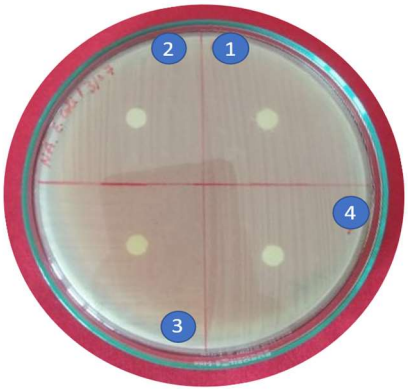
4.2 Evaluation of antimicrobial potential of *Tinospora cordifolia*

The antimicrobial activity of different extracts (ethanol, methanol, chloroform and aqueous) of plant part root, stem and leaves of *Tinospora cordifolia* were determined against *S. aureus*, *E. coli*, *P. aeruginosa* and *A. niger* by using disc diffusion method in the term of zone of inhibition.

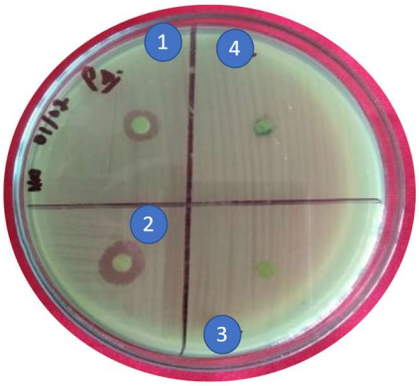
Table 4.10 Antimicrobial activity of roots in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia on* tested organisms

S.No.	Solvents	Zone Of Inhibition (mm)				
		Escherichia coli	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Aspergillus niger*	Overall mean
01	Ethanol	0.00 (1.00)	6.00 (2.65)	2.00 (1.73)	0.00 (1.00)	2.00 (1.54)
02	Methanol	0.00 (1.00)	7.00 (2.83)	3.00 (1.99)	0.00 (1.00)	2.50 (1.71)
03	Chloroform	0.00 (1.00)	4.00 (2.24)	0.00 (1.00)	0.00 (1.00)	1.00 (1.31)

A. Staphylococcus aureus



B. Escherichia coli



C. Pseudomonas aeruginosa

1. Ethanolic extract
2. Methanolic extract
3. Chloroform extract
4. Aqueous extract

Plate 5: Antimicrobial activity of roots in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia on* tested organisms (A, B, C).

04	Aqueous	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
	Overall mean	0.00 (1.00)	4.25 (2.17)	1.25 (1.43)	0.00 (1.00)	
		C.D.	S.E.$_{(d)}$			
	SOLVENT	0.05	0.02			
	TEST ORGANISM	0.05	0.02			
	INTERACTION	0.10	0.05			

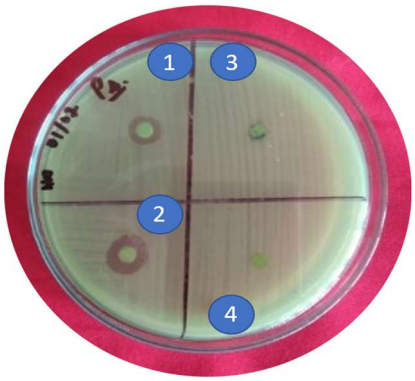
Figures in parentheses are square root transformed

Table 4.10 shows the antimicrobial activity of roots in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia* on *S. aureus, E. coli, P. aeruginosa* and *Aspergillus niger*. Based on above table it was concluded that the maximum zone of inhibition was found against *S. aureus i.e.,* 7.00mm and *P. aeruginosa. i.e.,* 3.00 mm. Pictorial description about the given discussion is shown in plate no. 5.

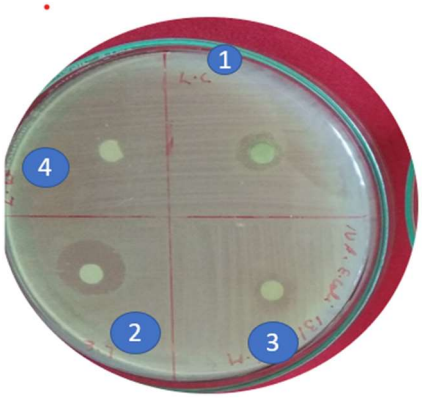
Table 4.11 Antimicrobial activity of stem in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia on* tested organisms

S.No.	Solvents	Zone Of Inhibition (mm)				
		Escherichia coli	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Aspergillus niger*	Overall mean
01	Ethanol	3.00 (1.20)	8.00 (2.99)	4.00 (2.23)	0.00 (1.00)	3.75 (2.06)
02	Methanol	5.00 (2.45)	10.00 (3.31)	5.00 (2.24)	0.00 (1.00)	5.00 (2.30)
03	Chloroform	0.00 (1.00)	3.00 (1.99)	0.00 (1.00)	0.00 (1.00)	0.75 (1.24)
04	Aqueous	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
	Overall mean	2.00 (1.61)	5.25 (2.32)	2.25 (1.66)	0.00 (1.00)	
		C.D.	S.E.$_{(d)}$			
	Solvent	0.08	0.04			
	Test organism	0.08	0.04			
	Interaction	0.16	0.08			

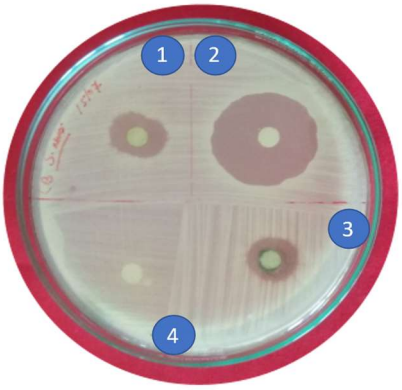
Figures in parentheses are square root transformed



A. Staphylococcus aureus



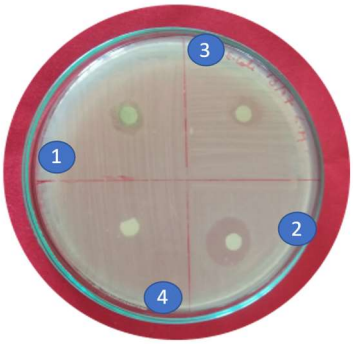
B. Escherichia coli



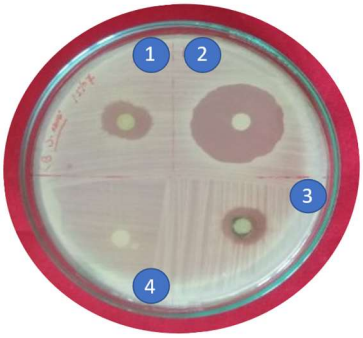
C. Pseudomonas aeruginosa

1. Ethanolic extract
2. Methanolic extract
3. Chloroform extract
4. Aqueous extract

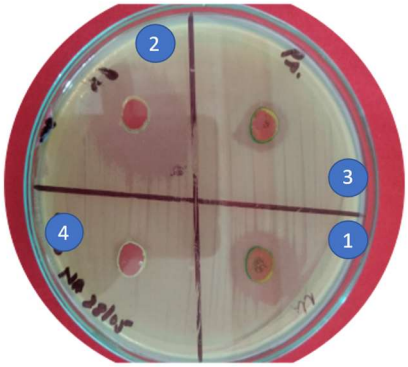
Plate 6 : Antimicrobial activity of stems in methanol , ethanol, aqueous and chloroform extract of *Tinospora cordifolia* on tested organisms (A, B, C).



A. Staphylococcus aureus



B. Escherichia coli



C. Pseudomonas aeruginosa

1. Ethanolic extract
2. Methanolic extract
3. Chloroform extract
4. Aqueous extract

Plate 7: Antimicrobial activity of leaves in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia* on tested organisms (A, B, C).

Table 4.11 shows the antimicrobial activity of stems in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia* on *S. aureus, E. coli, P. aeruginosa* and *A. niger*. Based on above table it was concluded that the maximum zone of inhibition was found against *S. aureus i.e.,* 10.00mm, *E. coli i.e.,* 5.00mm and *P. aeruginosa. i.e.,* 5.00mm. Pictorial description about the given discussion is shown in plate no. 6.

Table 4.12 Antimicrobial activity of leaves in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia on* tested organisms

S.No.	Solvents	Zone Of Inhibition (mm)				
		Escherichia coli	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Aspergillus niger*	Overall mean
01	Ethanol	3.00 (1.99)	5.00 (2.45)	4.00 (2.23)	0.00 (1.00)	3.00 (1.99)
02	Methanol	5.00 (2.45)	8.00 (2.30)	11.00 (3.47)	0.00 (1.00)	6.00 (2.50)
03	Chloroform	3.00 (1.20)	4.00 (2.24)	1.00 (1.48)	0.00 (1.00)	2.00 (1.67)
04	Water	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
	Overall mean	2.75 (1.85)	4.25 (2.18)	4.00 (2.03)	0.00 (1.00)	
		C.D.	S.E.(d)			
	Solvent	0.09	0.04			
	Test organism	0.09	0.04			
	Interaction	0.18	0.08			

Figures in parentheses are square root transformed

Table 4.12 shows the antimicrobial activity of leaves in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia* on *S. aureus, E. coli, P. aeruginosa* and *A. niger.* Based on above table it was concluded that the methanol extract gives the maximum zone of inhibition with *E. coli i.e.,* 5.00mm, *S. aureus i.e.,* 8.00mm, *P. aeruginosa* and *i.e.,* 11±00mm. Pictorial description about the given discussion is shown in plate no. 7.

After checking the antimicrobial activity of roots, stem and leaf with different extracts (Methanol, Ethanol, Aqueous and Chloroform) of *Tinospora cordifolia* on *S. aureus, E. coli, P. aeruginosa* and *A. niger* it is observed that the leaf methanolic extract get the maximum

zone of inhibition. Jeyachandran *et al.* (2003) reported that from methanolic, ethanolic, chloroform and aqueous leaf extract of *Tinospora cordifolia* methanolic leaf extract exhibited significant antimicrobial activity against, *P. aeruginosa*, *S. aureus* and moderate activity against *E. coli* and less activity against *Aspergillus niger*.

Table 4.13: Antimicrobial activity of methanolic leaf extract of *Tinospora cordifolia on* tested organisms.

S.No.	Leaves Methanolic solvent	Zone Of Inhibition (mm)				
		Escherichia coli	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Aspergillus niger*	Overall mean
01	Kotlangsan	7.00 (2.83)	5.66 (2.58)	11.50 (3.53)	0.00 (1.00)	6.04 (2.48)
02	Neri	5.00 (2.45)	7.00 (2.82)	11.00 (3.46)	1.33 (1.52)	6.08 (2.56)
03	Samloti	7.00 (2.83)	8.00 (2.99)	12.00 (3.60)	1.33 (1.52)	7.08 (2.73)
04	Manyiara	5.00 (2.45)	5.00 (2.44)	9.67 (3.26)	0.67 (1.27)	5.08 (2.35)
05	Jodan	7.00 (2.83)	4.00 (2.23)	10.67 (3.41)	0.00 (1.00)	5.41 (2.36)
06	Manwana	7.00 (2.83)	4.00 (2.23)	10.00 (3.31)	0.00 (1.00)	5.25 (2.34)
	Overall mean	6.33 (2.67)	5.61 (2.56)	10.80 (3.44)	0.55 (1.20)	
		C.D.	S.E.			
	Solvent	0.09	0.04			
	Test organisms	0.08	0.04			
	Interaction	0.19	0.09			

Figures in parentheses are square root transformed

From the table 4.13 it was concluded that the maximum zone of inhibition is found in location 3 *i.e.,* Samloti village in Kangra district. The zone is 7.00mm, 8.00mm, 12.00mm in *E. coli, S. aureus* and *P. aeruginosa respectively*. *E. coli, S. aureus* and *A. niger.* The zone of inhibition on tested organisms is 10±0.5mm on *E. coli,* 11±0.5mm *S. aureus* and 1±0.4mm on *A. niger.*

Table 4.14 Comparision of leaf methanolic extract with Gentamycin

S.No.	Test Microorganism	Zone of inhibition		Overall mean
		Leaf Extract(30µl/ml)	Gentamycin(30µl/ml)	

01	*E.coli*	7.00 (2.83)	11.00 (3.46)	9.00 (3.15)
02	*Staphylococcus aureus*	8.00 (2.99)	13.67 (3.83)	10.83 (3.41)
03	*Pseudomonas aeruginosa*	13.00 (3.74)	15.00 (3.99)	14.00 (3.87)
	Overall mean	9.33 (3.19)	13.22 (3.76)	
		C.D.	S.E.(d)	
	Test microorganism	0.14	0.06	
	Treatment	0.12	0.05	
	Interaction	0.20	0.09	

Figures in parentheses are square root transformed

Table 4.14 shows the comparison of leaf methanolic extract with gentamycin and zone of inhibition in *E. coli* is 7.00mm and in gentamycin is 11.00mm, *S. aureus* in 08.00mm in gentamycin is 14.00 mm and *P. aeruginosa* 13.00mm and in gentamycin is 15.00mm. Pictorial description about the given discussion is shown in plate no. 8. Simillarly Nagaprashanthi *et al.* (2012) has seen effect of gentamicin on tested organisms like *E. coli, S. aureus, P. aeruginosa* and observed that the zone of inhibition of 15mm in *E. coli*,17mm in *S. aureus* and 16mm in *P. aeruginosa.*

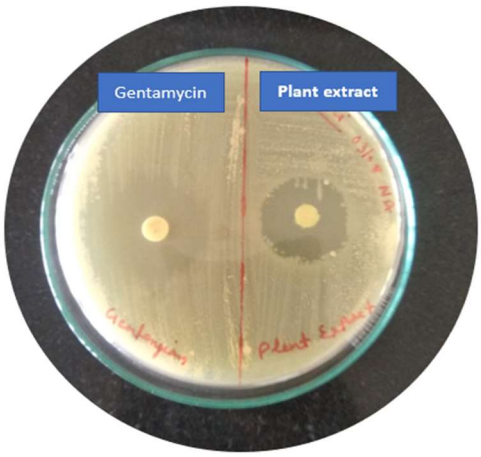
4.4 Determination of minimum inhibitory concentration (MIC)

MIC and MBC of plant extract were determined against *P. aeruginosa* (PA01), and *S. aureus* by standard microbroth dilution method according to National Committee for Clinical Laboratory Standards. MIC of plant extract was evaluated against *P. aeruginosa*, *S. aureus* by using microtiter plate assay described by *Webster et al.* (2010). Standard strain *P. aeruginosa* and *S. aureus* was cultivated in LB media under shaking conditions for 6 h to obtain a cell count of 10^6 cells/ml.

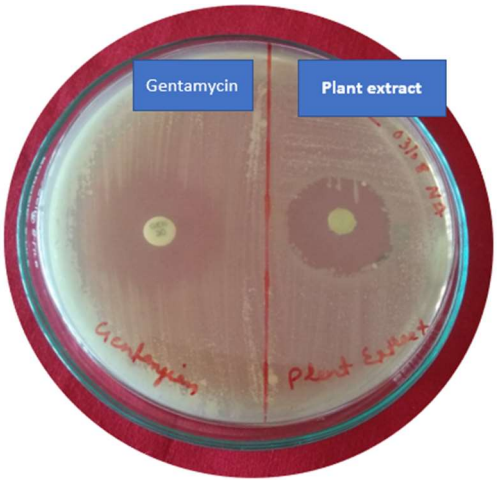
Minimum inhibitory concentration and minimum bactericidal concentration of leaves methanolic extract inhibited the growth of *P. aeruginosa* was observed and found to be the dilution (1:16) *i.e.,* 3.2 mg/ml and (1:8) *i.e.,* 6.4 mg/ml.

Minimum inhibitory concentration and minimum bactericidal concentration of leaves methanolic extract that inhibit the growth of *S. aureus* was observed and found to be dilution (1:8) *i.e.,* 4.7 mg/ml and (1:4) *i.e.,* 9.4 mg/ml, this is accordance with the study of (Pratihast

A. *Pseudomonas aeruginosa*



B. *Escherichia coli*



C. *Staphylococcus aureus*

1. Ethanolic extract
2. Methanolic extract
3. Chloroform extract
4. Aqueous extract

Plate 8 : Antimicrobial activity of leaves methanol extract (30µl/ml) of *Tinospora cordifolia* with Gentamycin (30µl/ml) on tested organisms (A, B, C)

et al. 2019) who have finds MIC and MBC of *Tinospora cordifolia*. (Mishra *et* al. 2013) suggest the MBC of *Tinospora cordifolia* was in the range of 1.29 mg/ml to 22.73mg/ml.

Chapter 5

Summary and Conclusion

Plants are the richest sources of drugs of traditional systems of medicine, nutraceuticals, food supplements (Hammer et al. 1999). The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. *Tinospora cordifolia* is a large deciduous climbing shrub found throughout India. The ayurvedic name of the plant is guduchi, giloy or amrita. In India, the extract of the plant is used as a remedy for many diseases including diabetes, hepatitis etc. The plant finds a special mention for its use in tribal or folk medicine in different parts of the country

Medicinal plants show properties due to presence of bioactive constituents which provide definite physiological action on the human body. The bioactive compound can be obtained from barks, leaves, flowers, roots, fruits and seeds. They are widely used in the medicine, agriculture, scientific research and countless other areas. The phytochemical analysis, antimicrobial activities of medicinal plants draw attention of plant researchers for standarazation of protocols for isolation and purification of these bioactive compounds and use them for pharmacological purposes.

Realizing this, the present study was done to find out the bioactive compounds present in *Tinospora cordifolia* from indigenous origin and to check their antimicrobial property the salient finding is achieved during the present investigation has been summarized under:

5.1 Quantification of bioactive molecules of *Tinospora cordifolia*

- Samples were collected from six villages of three districts (Hamirpur, Mandi and Manwana) of Himachal Pradesh
- Roots, stem and leaves were collected from each location.
- 04 types of extracts (ethanol, methanol, chloroform, aqueous) were used for study with plant parts roots, stems and leaves of *Tinosopora cordifolia*.
- HPTLC was done of all samples with solvent (ethanol:methanol) (50:50) ratio and all the RF value was calculated.

- In **aqueous extract** the root and stem of village Kotlangsan districts Hamirpur had lowest RF value i.e. 0.15 while leaf sample of same location had highest rf value *i.e.,* 0.90.
- In **ethanolic extract** the root and stem of village Manwana districts Mandi had lowest RF value *i.e.,* 0.05 while in leaf sample of village Neri district Hamirpur had highest RF value *i.e.,* 0.95.
- In **methanolic extract** the root of village Samloti districts Kangra had lowest RF value *i.e.,* 0.05 while leaf sample of same location had highest RF value i.e. 0.97.
- In **chloroform extract** the root of village Kotlangsan districts Hamirpur had lowest RF value *i.e.,* 0.04 while leaf sample Jodan and Manwana of districts Mandi had highest RF value *i.e.,* 0.96.
- By calculating all the RF values the highest RF value is of leaf methanolic sample of village Samloti district Kangra and this sample is selected for LC-MS.
- The LC- MS of leaf methanolic extract was done. The mass spectrometry pattern was studied and on the basis of peaks and molecular mass the following compounds were present in the leaf methanolic extract of *Tinospora cordifolia*. The compounds are berbrine, magnoflorine, menisperine, 20b-hydroxyecdysone, palmatine, 2-deoxy-20b-hydroxyecdysone and columbaine.

5.2 Evaluation of antimicrobial activity of *Tinospora cordifolia*

- Antimicrobial activity of various extract *i.e.,* methanol, ethanol, chloroform and aqueous roots, stems and leaves extract were tested using disk diffusion method against *S. aureus, E. coli, P. aeruginosa* and *A. niger*.
- Eighteen samples of roots, stem and leaves were collected from different villages of different districts in Himachal Pradesh.
- The antimicrobial property of root, stem and leaves methanolic, ethanolic, chloroform and aqueous extract were checked and compared and it was observed that the leaf methanolic extract had significant antimicrobial activity against tested organisms.
- The maximum antimicrobial activity was shown by leaf sample collected from the Samloti village of Kangra district, therefore that sample was compared with antibiotic i.e. gentamicin
- MIC of leaf methanolic extract against *S. aureus* and *P. aeruginosa* was observed and found to be 4.7mg/ml and 3.2mg/ml.

- MBC of leaf methanolic extract against *S. aureus* and *P. aeruginosa* was observed and found to be 9.4mg/ml and 6.4mg/ml.

Conclusion

In the quantification of bioactive compounds of *Tinospora cordifolia* through HPTLC it was concluded that there is a diverse range of bioactive compounds which are responsible for different medicinal properties of the plant. Methanolic extract showed the more number of bioactive compounds as compare to ethanol, chloroform and aqueous extract. In the evaluation of antimicrobial property of *Tinospora cordifolia* it can be concluded that *Tinospora cordifolia* has potential antimicrobial property. Methanolic leaf extract of *Tinospora cordifolia* showed greater inhibitory action than ethanolic, chloroform and aqueous root, stem and leaf extract against *S. aureus, E. coli, P. aeruginosa* and *A. niger*. The isolation and purification of bioactive compounds and evaluation of pharmalogical activities of isolated compounds of the crude sample are recommended as future research.

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Appendix – 1

1. Composition of Potato Dextrose Agar (PDA)

Composition	**g/l**
Dextrose	20.00
Agar	15.00
Potato infusion from	200.00

2. Composition of Luria Bertani Agar, Miller

Ingredients	**g/l**
Casein enzymic hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	10.00
Agar	15.00

3. Composition of Luria Bertani Broth Miller's Modifications

Ingredients	**g/l**
Casein enzymic hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	0.50

4. Composition of Nutrient Agar

Ingredients	**g/l**
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Agar	15.00

APPENDIX - 11

ANALYSIS OF VARIANCE

1. Antimicrobial activity of roots in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia on* tested organisms

Source of Variation	DF	Sum of Square	Mean Square	F-Calculated	Significance
Factor A	3	3.594	1.198	373.056	-0.00000
Factor B	3	11.098	3.699	1,151.974	0.00000
Intraction A X B	9	4.842	0.538	167.552	0.00000
Error	32	0.103	0.003		
Total	47	19.637			

2. Antimicrobial activity of stems in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia on* tested organisms

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Factor A	3	14.079	4.693	510.851	0.00000
Factor B	3	10.595	3.532	384.421	0.00000
Intraction A X B	9	6.035	0.671	72.988	0.00000
Error	32	0.294	0.009		
Total	47	31.003			

3. Antimicrobial activity of leaves in methanol, ethanol, aqueous and chloroform extract of *Tinospora cordifolia on* tested organisms

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Factor A	3	13.522	4.507	379.367	-0.00000
Factor B	3	9.912	3.304	278.093	0.00000
Intraction A X B	9	6.882	0.765	64.356	-0.00000
Error	32	0.380	0.012		
Total	47	30.697			

4. **Antimicrobial activity of methanolic leaf extract of *Tinospora cordifolia on* tested organisms.**

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Factor A	5	1.423	0.285	20.025	0.00000
Factor B	3	45.931	15.310	1,077.143	0.00000
Intraction A X B	15	1.867	0.124	8.756	0.00000
Error	48	0.682	0.014		
Total	71	49.904			

5. **Comparison of leaf methanolic extract with gentamycin**

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Significance
Factor A	2	1.610	0.805	64.454	0.00000
Factor B	1	1.485	1.485	118.910	0.00000
Intraction A X B	2	0.253	0.126	10.131	0.00265
Error	12	0.150	0.012		
Total	17	3.497			

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Title of the Thesis	**:**	**Quantification of bioactive molecules and evaluation of anti-microbial potential of *Tinospora cordifolia* Miers.**
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ABSTRACT

The present investigation on the **"Quantification of bioactive molecules and evaluation of antimicrobial potential of *Tinospora cordifolia* Miers."** were carried out in the Department of Biotechnology, College of Horticulture and Forestry (Dr. Y.S. Parmar University of Horticulture and Forestry), Neri Hamirpur (HP) during 2020-2021. *Tinospora cordifolia* is an angiosperm belonging to the Menispermaceae family and is a division of Magnoliophyta, class Magnoliopsida, and order of Ranunculaceae. It is a wide deciduous, glabrous, rapidly ascending shrub with several coiling branches extending approximately 3-4 feet in height and roughly 1 foot long. *Tinospora cordifolia* is a well-recognized and widely distributed traditional plant that is used successfully in Indian Ayurveda medicine. It has shown many promising biological activities such as antioxidative, antimicrobial, antihyperglycemic, anti-inflammatory, osteoprotective, hepatoprotective, antidiarrheal and antistress effects. It also contains many secondary plant metabolites, such as terpenes, alkaloids, flavonoids, steroids, and glycosides. The study was undertaken for evaluating the bioactive molecules and antimicrobial potential of *Tinospora cordifolia* collected from different districts of Himachal Pradesh. Analysis was done by using HPTLC of each root, stem and leave ethanolic, methanolic, chloroform and aqueous extract of samples taken from two villages of 3 districts. On the basis of RF value the districts Kangra sample was found to posses highest RF value *i.e.*, 0.97 and the sample with highest RF value was selected for LC -MS. In result found that 7 number of compounds were present. Those seven compounds are berbrine, magnoflorine, menisperine, 20b-hydroxyecdysone, palmatine, 2-deoxy-20b-hydroxyecdysone and columbaine which was responsible for the medicinal properties of *Tinospora cordifolia.* Considering the vast potentiality of *Tinospora cordifolia* as a source for antimicrobial drugs with reference to antibacterial, antifungal and anticandida agents, a systematic investigation was undertaken to screen roots, stem and leaves samples of *Tinospora cordifolia* collected from different Districts of Himachal Pradesh for its activity against various pathogens. Ethanolic, methanolic, aqueous and chloroformic extracts were tested but methanolic leaf extract of *Tinospora cordifolia* showed the maximum inhibitory activity against tested pathogens i.e *S. aureus, E. coli, P. aeruginosa* and *Aspergillus niger*. MIC of leaf methanolic extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was observed and found to be 4.7mg/ml and 3.2mg/ml. For future aspects, the isolation and purification of bioactive molecules and evaluation of their pharmalogical activity from the crude sample are recommended as future research.

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