Comparative Investigation of Secondary metabolites in *Cannabis sativa L.* from Pakistan

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A thesis submitted in partial fulfillment of the requirement for the degree of

Master of Science in Plant Biotechnology

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Co-Supervised by: Dr. Muhammad Qasim Hayat

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بسم الله الرحمن الرحيم

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Declaration

I certify that this research work titled "**Comparative investigation of Secondary metabolites in *Cannabis sativa L.* from Pakistan***"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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It is to confirm that MS thesis entitled "**Comparative investigation of Secondary metabolites in *Cannabis sativa L.* from Pakistan**" of **Ms. Rabia Bano** Reg No. **00000170962** has been examined by me. I undertake that,

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Copyright Statement

Dedication

Dedicated to my beloved parents and adored siblings, the one thing that never changes in my journey, the one thing I can always count on, is my family.

Acknowledgements

I am thankful to my Creator, **Allah Subhana-Watala** to have guided me throughout this work at every step and for every new perception which ALLAH setup in my mind to improve it. Indeed, I could have done nothing without ALLAH's guidance. Whosoever helped me throughout the course of my thesis, whether my parents or any other individual was His will, so indeed none be worthy of praise but ALLAH. Peace and blessings of Allah be upon last **Prophet Muhammad (Peace Be upon Him).**

I want to place my humble thankfulness to my honorable mentor, prestigious leader, my supervisor **Dr. Muhammad Tahir** for encouraging me to deal with issues related to my research during last two years. I can safely say that I have been extremely lucky to have a supervisor like him who responded to my questions and queries so promptly.

I feel my heartfelt gratitude towards my Co-supervisor, **Dr. Muhammad Qasim Hayat (HOD, Plant Biotechnology)** for his sincere guidance and motivations throughout my MS. His attention, constructive suggestions and cheerful enthusiasm helped me greatly throughout my experimental and thesis work. My success beholds your support.

I am thankful to you sir for sharing with us "The key to Success" that inspired me to achieve my goals. I am grateful to Principal ASAB, **Dr. Hussnain Janjua,** former Principal ASAB, **Dr. Peter John** and Rector NUST, **Lieutenant General Naweed Zaman** for providing us righteous place and resources to carry out the project.

I profound special thanks to my GEC committee members **Dr. Najam us Sahar Sadaf Zaidi** and **Dr. Rabia Amir** for their guidance and support.

This project would not have been completed without the assistance of my lab fellows. I am grateful for their moral support and wonderful company. I am blessed to have my best friend **Tehsin Fatima** and I want to take this an opportunity to place my thankfulness to her for standing by my side when the time gets hard, thank you for making me laugh when I did not even wanted to smile. There are no words to express my gratitude towards my brothers: **Dr. Muhammad Nauman Hussain, Muhammad Manan Hassan and Muhammad Arsalan** for their endless love, care, motivation and support for me.

I am profusely thankful to my **beloved parents**, **Mr. & Mrs. Chaudhary Muhammad Hanif** and my **paternal** & **maternal Family** members who raised me when I was not capable of walking and confided in me throughout every aspect of my life.

Rabia Bano

Table of Contents

Chapter no.1

INTRODUCTION

INTRODUCTION

Cannabis sativa L.

Cannabis sativa L. is a well-known herb which geographically originated from Central Asia . In ancient ages it was considered as folk medicine and used in textile fiber. Cannabis plant recently became prominent as a multi-task source with rapid growth cycle. It is not only the rich source of cellulose and wood fiber but also of the phytochemicals as well. Major areas include pharmaceutical industry, bioplastic manufacture, concrete like construction material and most importantly the secondary metabolites which are known to have bioactive potential for human health are obtained from this plant. Cannabis is popular because of phytochemicals with unique biosynthetic route i.e. cannabinoids, phenolic compounds and terpenes, having industrial interests including pharmaceuticals with therapeutic properties. Recent biotechnological prospects are employed to enhance yield and bioactivity of secondary metabolites obtained through plant tissue culture and genetic manipulation. Transcriptomics and metabolomics are the key technologies to enhance the yield and identification on large-scale. Cannabis also called hemp is promising crop due to its agricultural properties including pathogen and drought resistance, well-established root system which prevents soil erosion and lower water demands than other crops will allow to understands its chemistry and to explore its genome on molecular level through genetic engineering. Hemp is getting fame for its pharmacological potential against fetal diseases and scientists are encouraged to find some potential products from it. Use of oil, biomass, phytochemicals, trichomes and hairy root cultures highlight the bio-economy potential of Cannabis sativa (Andre et al., 2016). The plant with diverse chemical composition is famous to be used as a remedy for many ailments including gout and rheumatism, a potent sedative and

anesthetic also. Cannabis is collectively composed of almost sixty chemical constituents known as cannabinoids that add up to its biochemical activity are majorly synthesized by Cannabis metabolism through liver enzymes with no known effects. The most important constituent derived from Cannabis is 9- tetrahydrocannabinol (THC), is popular for its greater psychoactive potential, rapid metabolism potential lipophilic nature. Its lipophilic nature helps to diffuse through membrane barriers made up of phospholipids and hence make it able to cross membranes of brain tissues (M Liu et al., 2010). Cannabis is categorized as medicinal plant into drug-type which is enriched with psychoactive cannabinoid Δ^9-THC to be used for cure and recreational purpose and later is fibre-type being devoid of THC and enriched with cannabidiol related constituents to be used in textile and food industry but cannabis is popular all over the world owing to its drug-type as a well investigated plant having medicinal values against many diseases and as a primary source of therapies as well.

Morphology of *Cannabis sativa L.*

Cannabis sativa L. belongs from genus of the flowering plant of family Cannabaceae consists of three disputed species i.e. *Cannabis indica*, *Cannabis sativa*, and *Cannabis ruderalis* and the last one is considered to include in C. sativa . C. sativa is major undivided species consisting of the sub species as mentioned above (Joppa et al., 2010).This genus is biogeographically originated from central Asia including south Asia (Lambert et al., 2009). C. sativa is annual, dioecious and an angiosperm herb. It contains digitate or palmate compound leaves with serrate leaf-lets (Asenso et al., 2010).There is a single leaf-let in the first leave pair and then it increases gradually to about thirteen leaf-lets per leaf owing specie variety and growth conditions provided. Again the tip of flowering pant consists of single leaf-let per leaf and lower pair of leaf occurs in alternate leaf arrangement lading towards the main stem of mature plant body. Leaves show pattern of peculiar

and the diagnostic venation which aids in distinguishing Cannabis leaves from species that are not related but have similar leaf pattern. Pattern of serration, as in common , originates from bottom and keeps on extending towards tips.

Plantlets of Cannabis can also be precisely examined through microscopy of the leaf cells and other features when one have expertise and equipment (Watt et al., 1962). As we know it is a dioecious specie having individual male and female unisexual flowers which show sexual dimorphism i.e. male plant is slender and taller than the female one and have shorter life cycle. The unisexual flowers are usually terminal during early stages and lateral at later stages. Male inflorescence contains hanging panicles which is branched having no or few leaves and number of variable flowers. These flowers have a perianth of 5 sepals which enclose androecium, consists of 5 stamens with subtle stalks. At maturity the anthers undergo longitudinal dehiscence and release pollen grains , being widely spread by wind (Hammond et al., 1977). The female inflorescence is known to be raceme developing at apex of plant also at the leaf axils or the lateral branches. These female flowers are very simple in structural composition having green bracts covering ovary and perianth completely. This flower is uniloculate has short style which distally differentiates into a bifid stigma. *Cannabis sativa* contains ten chromosomal pairs with 9 autosomal and one sexual chromosomal pair. XY endows male and XX denotes the female one. In Cannabis, sex determination is done through X-autosomal dose than on Y- mechanism (Grant et al., 1994).

Figure 1.1: Figure shows difference between male and female cannabis plant.

Figure 1.2: figure contains flowering male (A) and female seed bearing (B) plant of Cannabis with actual size. Male flower(1) with enlarged details, pollen sacs from various angles (2 and 3), pollen grain (4), female flower with the cover petal(5), female flower (6), without cover petal; female fruit cluster (7), longitudinal portion ; cover petal; the same without cover petal (9,10), same in cross-section (11); same in the longitudinal section (12); seed without hull (13) Makwana, R. (2007).

Secondary Metabolites in *Cannabis sativa L.*

Cannabis plant have been popular as a common wild source of traditional and active form of medicine always. It is ubiquitously known as traditional medicine in various cultures. The products obtained from Cannabis plant including all forms such as marijuana, hash oil and hashish are prominent medicinal agents. Cannabis is enriched with almost 60 phytochemical constitutes majorly cannabinol, tetrahydrocannabinol, cannabigerol,cannabidiol and cannabichromene. These phytochemicals have strong pharmacological profile and also have great potential for therapeutics such as cannabinoids are known to treat hypertension, epilepsy, asthma and many more (Asati et al., 2017). The all chemical constituents extracted from Cannabis sativa are collectively known as phytocannabinoids. Unique alkyl resorcinol and monoterpene groups of secondary metabolites are produced in Cannabis sativa and the best constituent i.e. tetrahydrocannabinol (THC) has a G-protein specific receptors called as cannabinoid1 and 2 (CB1 and CB2) receptors re known to represent induction of potential transient psychotic reaction in previously isolated compounds (Morrison et al., 2009). Δ 9 - tetrahydrocannabinolic acid is also primarily a psychoactive agent produced in acidic in glandular trichome of bracts and is carboxylates with the passage of time or heated to the Δ 9 -tetrahydrocannabinol (THC) (Mechoulam., 2005 & Pertwee., 2006). The extracts is enriched with more chemicals such as cannabigerol which have been exploited to modulate THC effects (ElSohly et al., 2005). The cannabinoids are those components which are not responsible for receptor activation. Another metabolic compound nabilone is prior domperidone, alizapride and prochlorperazine to control vomiting and nausea during cancer chemotherapy and is very popular as a drug in Canada (Amar., 2006).

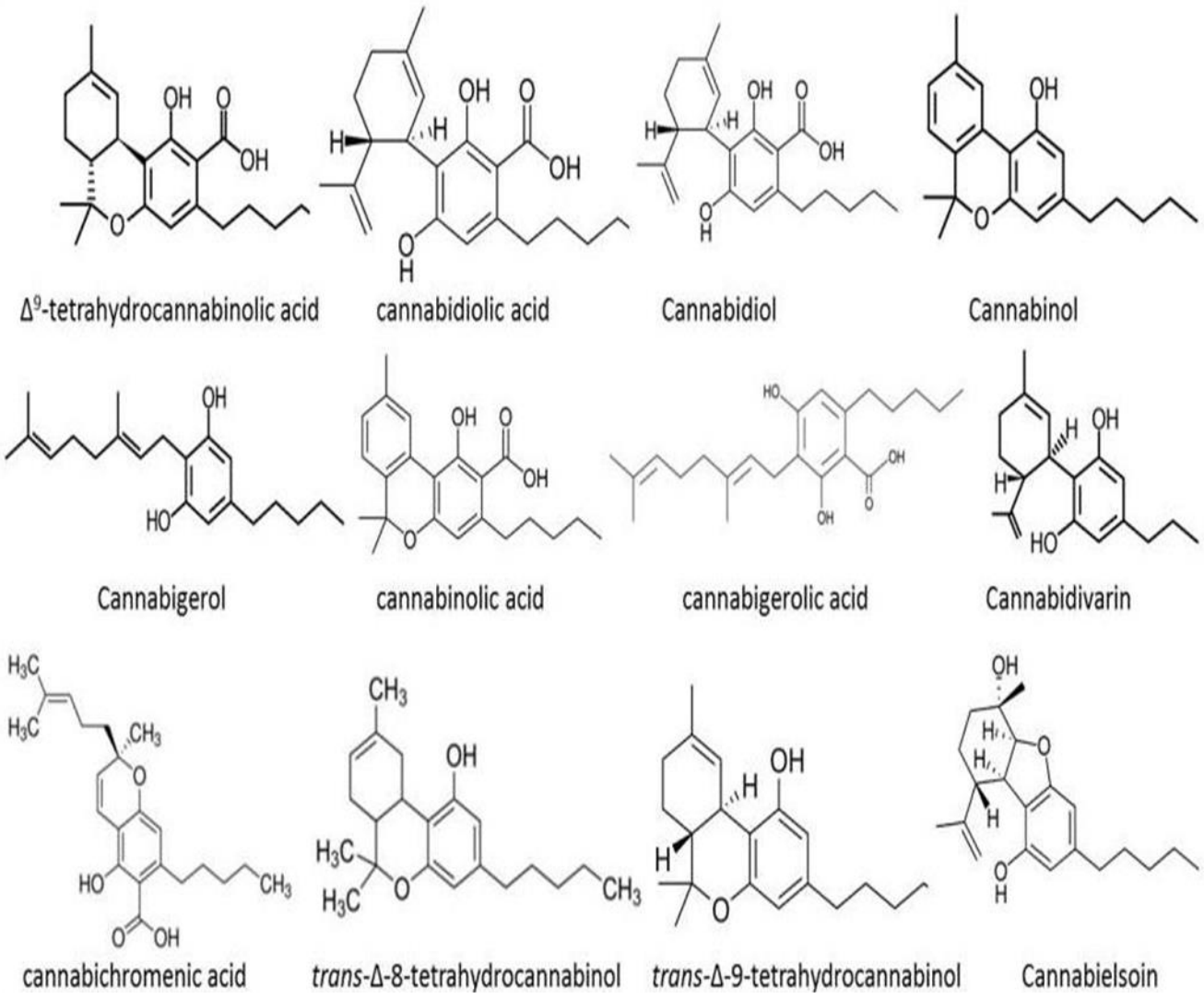


Figure 1.3: Figure shows major secondary metabolites of Cannabis sativa along with their structures.

Chapter no. 2

LITERATURE REVIEW

LITERATURE REVIEW

Many medicinal plants used for many purposes such as, synthesis for paper, clothes and for the prevention of many diseases (Nasrullah et al., 2012 & Quideau et al., 2011) . Normally cannabis is dioecious wind pollinated , annual herb of the family Cannabaceae, monoecious plants occur in some populations that has also been used in different regions of the world as medicinal plant providing a source of fiber, oil, food medicine and paper (Williamson & Evans, 2000). *Cannabis* is the primary ingredient to produce hemp Hashish and marijuana. It has been used by innumerable ethnic societies in Asia. Throughout many years *Cannabis* has been widely used as illegal drug. Potency of cannabis preparation is increasing (Lovestead et al., 2017). *Cannabis different* parts has been used for therapeutic purposes for curing different diseases of mankind (Sarfaraz et al.,2008). This genus has three species *C sativa C indica and C ruderalis. Cannabis sativa* and *cannabis indica* are grown all over the world but in Pakistan *Cannabis* grow as wild crop thy flourish right across northern Punjab up in Khyber-Pakhtunkhwa and are well established in green belts in Islamabad (Anwar et al., 2006). This crop is commonly called as bhang cultivated at a low scale in different areas of Pakistan due to illicit reasons.

Cannabis, Hemp and Marijuana

Marijuana formed from dried flowering tops leaves stem and seeds of cannabis sativa. It is used as psychoactive recreational drug. Conflicts regarding the legitimacy of medicinal marijuana use extend even the even to the level of state versus federal law. Popular conception seems to be that marijuana is harmless pleasure, access to which should not be regulated or considered illegal. Marijuana is mostly used drug in United States (Han et al., 2015). Recent times have blessed it with the little leading psychoactive plant available. Hemp is characterized by low amount of psychoactive compound tetrahydrocannabinol in their leaves and flower. From many years

industrial hemp is grown by more than 30 countries worldwide for fiber seed and oil and other products including food.

Cannabis in ancient times

Origin of cannabis farming or cultivation is from china where it was used in practice of shamanism, nomadic tribes carried it to western Asia and India Cannabis were also used in Assyria, Babylon and Palestine linking cannabis to Jewish religion (Li, H.L., 1973). It was used to relief patients of stress and anxiety. With time abuse of mature plant parts resulted in decisions of completely obliterating its cultivation.

ETHNO BOTANY

Term ethnobotany means study of interaction between plants and humans this not essentially mean how plant usage by other people. Medicinal plants study is not limited to local cultures. Relationships of plants and indigenous cultures is clear by usage of ethnobotany in plants collection (Iwu, M. M. (2014). Plant based medicines are preferred in the world from decades, local societies of developing countries rely on herbal medicines it's a great link of plants and humans. It provokes to ethnobotany (Ugulu et al.,2009). Pakistan has great biodiversity of medicinal plants therefore Pakistan has great ethnobotanical importance with traditional usage of plants by tribal communities (Ahmad et al., 2014) Cannabis sativa known due to its use as psychoactive, medicinal value, fiber producing plant and plant use for food. In Pakistan local societies use cannabis sativa as herbal medicinal plant and first time use for the cure of respiratory disorders in Northern areas (Kayani et al., 2014). Cannabis sativa L. is one of the oldest food, fibre, medicinal, psychoactive and oil plants known. It has been used by innumerable ethnic societies in Asia. Uttaranchal (India) is an ethnic region where the plant is a part of the local culture. In this paper the indigenous uses and ethnobotany of its seed, seed oil, stems, fibre, leaves, inflorescences

and resin along with various recipes of seeds are described. A theory of its introduction to Uttaranchal by ethnic races is also given. It is concluded that in the light of the present commercial and industrial uses of Cannabis its cultivation should be promoted in Uttaranchal and other parts of the Himalayan regions of India, where it grows naturally and is cultivated for folk uses (Rand et al., 2003).

Cannabis and therapeutics

Obesity

Excessive amount of fat accumulate in body leads to obesity higher amount of omega-3 and omega-6 reduce body activity. A person is obese when their body mass index is 30 or greater. From decades *Cannabis* popular to increase appetite and food consumption. Several brain regions contain higher level of cannabinoid 1 receptor (CB1) (Lauckner et al., 2008). Cannabinoid-1 receptor activity linked with food intake enhancement and reduced obesity (Alshaarawy et al., 2019). Long term use of cannabis related with decreased risk of obesity (Scheffler et al.,2018). Consumption of cannabis lower down energy storage level and elevate metabolic rate results in lower obesity and body mass index (Bauwens et al., 2018).

Cancer

From last year's cannabis sativa got attention due to its compounds, such as $\Delta 9$tetrahydrocannabinol and cannabidiol and their effects on inflammation and pain related to cancer. (Ongaro et al., 2015). This study also provides information about medical cannabis for cure and management of cancer symptoms. Cannabinoids due to its antiemetic and analgesic effects have been allowed in the pain reliever medicine their anti-cancer. The therapeutic potential of cannabis and cannabinoids with extensive anti-cancer capabilities was discovered in last decade. Preclinical and clinical trials provided evidence (Sledzinski at el., 2018). Cannabinoids have anti-

cancer activities it was notable in lung adenocarcinoma model and further studies reveals in vitro and in vivo glioblastoma multiforme, breast prostate, thyroid, colon, skin, pancreatic tumor growth inhibition. Proliferation of cancer cells inhibited by inhibiting the effects of adenylyl cyclase and cAMP/protein Kinase A proliferative pathways (Pisanti et al., 2009). As Cannabinoids perform anti-cancer activity as a single agent, they may useful compounds when bind to other cytotoxic factors, strategy of cannabinoids to bind with other compounds and induce cell death through signal transduction pathway is a good scientific approach (M Liu at al., 2010).

Neurological Disorder

Cannabis sativa used as medication for epilepsy to date, cannabinoids have been allowed in the palliative medicine due to their analgesic and antiemetic effects, but increasing number of preclinical studies indicates their anticancer properties. Cannabinoids exhibit their action by a modulation of the signaling pathways crucial in the control of cell proliferation and survival. Many in vitro and in vivo experiments have shown that cannabinoids inhibit proliferation of cancer cells, stimulate autophagy and apoptosis, and have also a potential to inhibit angiogenesis and metastasis. In this review, we present an actual state of knowledge regarding molecular mechanisms of cannabinoids' anticancer action, but we discuss also aspects that are still not fully understood such as the role of the endocannabinoid system in a carcinogenesis, the impact of cannabinoids on the immune system in the context of cancer development, or the cases of a stimulation of cancer cells' proliferation by cannabinoids. The review includes also a summary of currently ongoing clinical trials evaluating the safety and efficacy of cannabinoids as anticancer agents (Grotenhermen, F. 2004).

Schizophrenia

Schizophrenia is a serious mental disorder, it appears with both positive and negative symptoms (Taksande et al., 2011). 1% adult population is affected by schizophrenia research on cannabis and schizophrenia started from 1990s, it is reported that Cannabis sativa is more likely used by schizophrenia patients (Thornicroft, 1990) and existing mental disorder is associated with continuous cannabis use (Linszen et al.,1994.,Grech et al.,2005). Life time cannabis use mediates changes in function and biochemistry of brain which are the symptoms of schizophrenia (Shiozawa et al., 2008).Over the past few years five endogenous ligands and endogenous cannabinoid [CB1 and CB2] receptors has been identified. Several researchers gave evidences which suggesting alterations in endocannabinoid system in several patients which may contribute in schizophrenia disorder in these patients (Kirsten et al., 2014).

Epilepsy

Epilepsy is not contagious neurological most common disease, especially in third world countries. Epilepsy patients have seizures, most of the patients suffering with this disease have drug resistance epilepsy (Hussain et al., 2018). Cannabis use as drug treatment is becoming more prevalent. Modern neurologists using cannabis for the treatment of seizures (Gordon et al., 2001). When the recommended medicines useless to controls the children's seizures then parents started to use alternative treatments one of these treatments is cannabidiol present in cannabis (Izzo et al., 2009).

Asthma

Asthma is more common chronic disease all over the world, conventionally it is categorized as allergic reaction where this atopic Reaction cause reversible obstacle of ventilation providing

duct (Vuolo et al., 2015). Cannabis have several compounds one of them is tetrahydrocannabinol which is useful or asthma patients. In medicine, researchers were elucidating whether cannabis can cure asthma or not depending upon the anti-inflammatory properties of cannabis as asthma is inflammatory. Further studies also proven the importance of cannabis by the virtue of its tetrahydrocannabinol constituents to open up respiratory passages which control coughing being antagonistic to smoking. Smoking alone restricts the respiratory tract and is regarded as harmful for asthma patients to cause adverse health effects but last two decades studies have found that the asthma patients with low level cannabis intake have dramatically improved the functioning of their lungs by lagging behind the chronic pulmonary damages and vice versa with higher cannabis dosage (Bramness et al., 2019).

Chapter no.3

MATERIALS & METHODS

Sample Collection

Tradition of medicinal plant collection is very old. The sampling for the plant carried out in period of October to November. *Cannabis Sativa .L* is identified by the department of NARC. Fresh and disease free plants were collected from hilly area of NUST sector H12 Islamabad and plains of Mailsi and Fateh jang. The plants were stored in zip locker bags for further analysis.

Herbarium Preparation

A herbarium is a storehouse where dried specimens of plants stored in form of database. After collection pressed dried plant sample is mounted on a sheet called herbarium specimen. Herbarium labeling Information consists of date, collector name and location written on sheet. Herbarium specimen were prepared according to standard protocol of Pakistan museum of natural history. Prepared Herbarium sheets of *Cannabis sativa* plant submitted to Pakistan museum of natural history accession numbers assigned by PMNH to each herbarium sheet for future reference.

Molecular Identification of plant material

DNA extraction

DNA extraction is a main crucial procedure in molecular identification of plant material. DNA extracted from *Cannabis sativa* leaves by cetyltrimethylammonium bromide based method described by Doyle & Doyle in 1987. Leaf material was washed with ethanol and grinded in mortar and pestle by using liquid nitrogen. Grinded material transferred in 1.5 ml eppendorf tube. 1ml CTAB buffer Preheated in shaking incubator at 65 degree was added to eppendorf having grinded plant material. A pinch of PVPP (polyvinyl polypyrrolidine) also added to plant material. Eppendorf having grinded plant material, CTAB and PVPP put into shaking incubator at 65 degree for 30-60 minutes and was mixed after a few time. After incubation this material shifted into two

1.5 ml tubes and put in fume hood for cooling down for 5-8 minutes. Equal amount of chloroform and isoamyl alcohol in 24:1 ratio were added in both eppendorf tubes and mix slurry . Tubes were centrifuged at 9000 rpm for 10 minutes or 15000 rpm for 5 minutes by centrifuge to separate the phases. Removed the aqueous phase (supernatant) with pipette and transferred to an autoclaved 50 ml falcon tube. Added equal volume of cold isopropanol covered the tube and gently mix by inverting and was incubated for 1 to 2 hours at -20^{o}C or overnight at room temperature. Then Centrifuged at 9000 rpm for 10 minutes at room temperature Pellet was saved and supernatant was removed. I ml of cold wash buffer added directly to pellet and agitated it gently and incubated for 30 minutes at room temperature. Again pour off supernatant and pellet was dried in incubator at 37 ^{0}C. The pellet was dissolved in 1 ml TE buffer and store at -20^{o}C for future use.

Gel Electrophoresis

The quality and quantity DNA was analyzed through gel electrophoresis using 1% agarose gel. The 5 µl sample of DNA 1 µl of 6X Thermofisher Scientific loading dye was mixed thoroughly by using pipette. The mixture was loaded into the designated well on the gel. The 100 bp Thermofisher Scientific DNA ladder was used gene ruler. TE buffer as negative and the DNA of known sample as positive sample was also loaded in their respective wells to validate the integrity of the results. The electrophoresis was performed by supplying 300 mA current while 80 V was provided to create electric gradient in the gel tank. After electrophoresis, the gel with results was analyzed under Ultraviolet (UV) light provided by UV Trans illuminator (Wealtech) and image of the gel was taken using Gel Documentation system for further record.

PCR Amplification

Internal transcribed spacer region of *Cannabis sativa* was amplified. This amplification was done by using set of ITS (universal) primers. PCR reagents were put in a PCR tubes and this tube vortex for mixing the reagents. The reaction mixture was contained total volume of 25µl in PCR tube. 1st of all different ranges of temperature starting from denaturation to extension were set in according to given ITS primers region to amplify given sample of cannabis. PCR profile set at the temperature for denaturation step was 94ºC for 30 seconds minutes, 50ºC for 30 sec was primer annealing temperature. Temperature required for annealing was 72ºC for 1 minute and termination was done 5 minutes at 72ºC. Reaction was consist of 35 cycles. Standard PCR reagents used for the reaction are as follows.

Table 1.1; Recipe for ITS amplification is given below.

Sr	Reagents	Concentration	Volume
1	PCR water		14.03µl
2	MgCl2	50mM	1.5µ
3	10X Buffer		25µl
4	Taq Polymerase		0.2µl
5	DMSO		1µl
6	BSA		1µl
7	Reverse Primer	10µM	0.5µl
8	Forward primer	10µM	0.5µl
9	dNTPs	2mM	2.5µl
10	DNA template	100ng/ml	1µl
11	Total volume		25µl

Sanger Sequencing

The confirmed and cleaned PCR amplicons were send to Macrogen Korea with all the required details for Sanger sequencing and Phylogenetic Analysis.

Phytochemical analysis

Preparation of extracts

Drying and Grinding

Plant material was air dried at room temperature for 5-7 days. The drying was successful as after seven days when the stems of *Cannabis sativa* were broken with a sound of tick fresh green color of leaves changed to darker tone. Leaves were separated and grinded into a fine powder that could sieved through mesh size eight.

Maceration

Maceration is a method which has been adopted and widely used to break the walls of cells, to separate the bioactive compounds. Equal amount of powdered leaf material of *Cannabis sativa* 1:10; 1g were dissolved in three different type of solvents aqueous, ethanol and methanol. Volume of each solvent were 10 ml. This mixture was put in dark and stoppered containers allowed to stand in dark for a period of 15 days. Samples were shaken manually for 10-15 minutes twice a day.

Rotary Evaporation

As the plant extracts were getting prepared with three solvents

1. Ethanol
2. Methanol
3. Distilled water

As a result the temperature of Rotary Evaporator was adjusted accordingly on 78^{o}C for ethanol, 64^{o}C for methanol and 100^{O}C for distilled water. To concentrate the extract filtrate was added to round bottom flask which was fixed on rotary evaporator. At a certain desired concentration filtrate was removed and air dried on petri plate in fume hood.

Solvent Extraction

To remove the chlorophyll chloroform extraction performed. Dried extract was measured by weight balance. Total weight of each dried extract was 15 grams. This dried extract again dissolved in the relevant solvents. Separatory funnel fixed on stand mixture was put in this funnel and chloroform was also added in separatory funnel with stopcock at bottom closed. Extract and chloroform blended 3 times for 30 seconds with the gap of 30 minutes, and leave the funnel on stand for few minutes. Mixture was separated into two immiscible phases, each phase has different densities. The lower layer containing chlorophyll released from the bottom upper chlorophyll free extract phase was put into petri plate which transferred in fume hood for air drying extract to evaporate chloroform. Dried extract was saved for further analysis.

Plant Extract Characterization

Exactly 20g dried powdered of *Cannabis sativa* was macerated separately in 200 mL of ethanol, methanol in dark colored glass bottles. Aqueous extract were also prepared by soaking same amount of plant material in water (Njume et al., 2011).

Qualitative Phytochemical Tests

Cannabis Sativa get attention due to possible presence of potentially active classes of compounds, such as alkaloids, phenols, anthraquinones ,anthocyanin, flavonoids, carbohydrates proteins etc (Schreier et al., 1976).

Alkaloids

Alkaloids were determined by adding few drops of hagers' reagent in 500 µl of ethanol, methanol and aqueous extract of *Cannabis sativa* leaf in a test tube resultant mixture was observed having yellow precipitates. It is previously reported that alkaloids are soluble in methanolic and aqueous extracts (Dhawan et al., 2017). Ethanol is also effective to extract alkaloids (widyawati et al., 2014).

Phenol

1% FeCl3 was mixed with 500µl of every Ethanol, methanol and aqueous extract of *Cannabis sativa* in test tube, bluish black coloration confirmed presence of phenols. Phenol presence is already confirmed in ethanol, methanol, and aqueous extracts of *Azadiracta indica, Ocimum sanctum, and Momordica charantia* leaves (Rafat et al., 2017).

Flavonoids

For detection of flavonoids crude extracts of methanol, ethanol and aqueous were treated with lead acetate formation of yellow precipitates indicate the presence of flavonoids. This Phytochemical also reported in Periploca aphylla (Garg et al., 2008).

Anthraquinones

To 3ml extract dissolved in same concentration of benzene then added 5ml ammonia into it, this resulted in appearance of pink, violet color, which confirmed the anthraquinones. Anthraquinone presence in ethanol and aqueous extracts were already reported in *Cannabis sativa* (Akter et al., 2016).

Anthocyanin

2ml HCl mix with 2ml extract in test tube then few drops of ammonia added into it. Same process repeated for all three extracts ethanol, methanol and aqueous. Presences of anthocyanin confirmed by the appearance of bluish violet coloration in methanolic extract. There were no appearance of pink or bluish violet coloration in ethanolic extract. Anthocyanin were not present in *cannabis sativa* ethanolic extract. It was present in aueous extract. Anthocyanin already reportd in Daphne mucronata Royle extracts.(Zeb et al 2016).

Leucoanthocyanin

To observe the presence leucoanthocyanin 500$\boldsymbol{\mu l}$ of extract mixed with 500µl isoamylalcohol organic layer truns not turned into red it reveals the absence of leucoanthocyanin in ethanol, methanol and aqueous extracts (Cheikhyoussef et al., 2015).

Tannins

First, about 1 ml of the ethanol extract was added in 2 ml of water in a test tube. 2 to 3 drops of diluted ferric chloride solution was added and observed for green to blue-green (cathechic tannins) or a blue-black (gallic tannins) coloration. Second, 2 ml of the aqueous extract was added to 2 ml of water, a 1 to 2 drops of diluted ferric chloride solution was added . A dark green or blue green coloration indicates the presence of tannins (Zohra et al., 2012).

Phlobatanins

Precipitate test was performed by adding 2ml of extract in test tube and then 2ml of 1% HCl were mixed into it and boiled by putting test tube in water bath. Red precipitates were formed in methanolic and aqueous extracts and no precipitates were formed in in test tube having ethanolic

extract. Methanolic and aqueous extract of A. Jackson medicinal plant of family Ranunculaceae also have Phlobatanins which is reported in paper (El-Swaify et al., 2017).

Coumarins

2ml of Ethanolic methanolic and aqueous extracts of Cannabis sativa were added in test tube separately and 3ml of 10% NaOH were added in each test tube yellow color were appear in test tube which is indication of Coumarins presence. Presence of Coumarins in medicinal plants extracts were already reported Ghosh et al., 2015).

Terpenoids

Terpenoids metabolites stimulate development and growth and protect from biotic and abiotic environments in plants. They also play a major role in pharmaceutical industry. Test was performed to check the Terpenoids in all three types of extracts. 2ml of extract were added in to test tube ethanol and 2ml of $CHCl_3$ were added into it and heat it then 3 drops of CH_2SO_4 were added in test tube extract change into deep red color. Extract of Valeriana wallichii DCV also have Terpenoids which is reported (Subhan et al., 2010).

Sterols

Salkowski's test were performed to check the steroids in Cannabis sativa leaves extracts. 1ml of extract were in test tube and few drops of concentrated H_2So_4 were added into it boiled on burner and put on stand red color appear in lower layer in test tubes having ethanolic and methanolic extracts. No color were appear in aqueous extracts of cannabis sativa leaves. Steroids already reported in medicinal plant ethanolic and methanolic extracts (Ndam et al, 2014).

Steroids

Steroids are main secondary metabolites in plants. They have best anti-inflammatory properties due to which they are widely used in treatment of different diseases like arthritis and lupus. The extract of Cannabis sativa leave extract contains sample have steroids which was observed by performing Salkowski's test. Each of the extracts showed reddish brown color at the interface, which shows strong presence of steroids in the extract from all three localities. It is already reported in literature (Hussein et al., 2014). Presence of steroids in ethanolic and Methanolic extract were reported in other medicinal plants (Kaladhar ET AL., 2014).

Saponins

Saponins are bioorganic molecules and aglycone it has various biological medicinal and pharmaceutical properties. Medicinal plant have Saponins. Foam test were performed to analyze the presence of Saponins in Cannabis sativa leaves extract from three different localities. 2 ml of extract were added into 2ml of H_2O and heated it, extract gives froth appearance. Saponins were present in methanolic extract reported (Verma et al., 2013)

Resions

In folk as well as modern world's medicines resins used due to its bioactive and pharmaceutical properties. 2ml of extract, 3 ml of acetone and 3ml of HCl add in test tube and heat for 30 minutes. Pink coloration change into red. This test performed for all three extracts of Cannabis sativa. Medicinal plants leaves extracts have resins which is confirmed (Lee et al., 2017).

Glycosides

2 ml of acetic acid, 2ml of Cannabis sativa leaf extract and 2ml of $CHCl_3$ were added into test tube violet to blue or green color of extract changed. This method for test were confirmed from medicinal plant paper (Gul et al., 2017).

Emodins

Cannabis Sativa leaf extract were phytochemically screened for all three type of extracts. 2ml of extract, 2ml of ammonium hydroxide and 2ml of benzene were mixed in test tube extract having emodins changed into red in color (Dubey et al., 2019).

Aminoacids

Ninhydrin test were performed to check the presence of amino acids from *Cannabis sativa* leaf extracts. 1ml of extract were taken in test tube and few drops of ninhydrin added into it. Extract color changed into violet (Ansari et al., 2016).

Proteins

Xanthoproteic test were performed to analyze the presence of proteins in *Cannabis sativa* leaf extract. 1ml of extract and 1 ml of concentrated hydrochloric acid were added in test tube and boiled white precipitates changed into yellow. Presence of proteins in medicinal plants already reported in paper (Karthishwaran et al., 2010).

Carbohydrates

Fehling test performed to confirm the presence of carbohydrates in *Cannabis sativa* leaf extracts. *Abrus precatorius* is medicinal plant its ethanolic extract have carbohydrates (Arora et al., 2011). Methanolic and aqueous extract also have carbohydrates (Ram et al., 2015).

Antioxidant Activity

Antioxidants are molecules which are scavengers of free radicals such as super oxides and nitrogen per oxides and inhibits the initiation of oxidizing agents. These free radicals are produced in the cells of organism by any type of stresses and damages. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) is steady free radical by which delocalization of extra electron over the atom all in all. As the molecule does not dimerize. (Alam et al., 2013). Through DPPH method we can evaluate the antioxidant potential of compound, biological source or extract (Vijayalakshmi et al., 2011). Free radical scavenging activity of ethanolic, methanolic and aqueous extracts of *Cannabis sativa* evaluated through DPPH method by following the reported method with some modifications (She et al 2010). So, stock solution prepared, 1 mg of semi-solid extract dissolved in their respective solvents, methanol ethanol and water. Five serial dilutions 10 µg, 25µg, 50µg, 75µg, 100µg were made using respective solvents. When extracts were mixed with DPPH reagent solution color were changed from purple to yellow. Methanol were used as a blank. Absorbance were measured at a wavelength of 517nm through spectrophotometer. DPPH percentage of reactive molecule scavenged by extract were measured. DPPH is light sensitive chemical dealing in dark is mandatory for positive results.

Chapter no. 4

RESULTS

Phytochemical screening

Extract was prepared in three different type of solvents ethanol, methanol and aqueous. Phytochemical screening of extracts of three different localities including Multan, Islamabad and Fateh Jang was done using different phytochemical tests. Different type of compounds were analyzed in all type of extracts and variation also observed due to different localities. The whole process used for screening was given as follows:

Drying and grinding of Plant material

Female plant samples of *cannabis sativa* were collected from three different localities Multan, Islamabad and Fateh Jang. Samples were dried in shade and leaf was separated from the plant and grinded.

Figure: Cannabis sativa plant

Figure: Grinding of Cannabis sativa leaves

Maceration

10 gram grinded *Cannabis sativa* leaf powder dissolved in 100 ml of solvent. Aqueous ethanol and methanol were selected. 10 gram of each three localities powder were separately dissolved in solvents in dark bottles. Every day bottle was shaken for 10 minutes two times in a day. After two weekes maceration was compeleted

Figure Maceration of Cannabis sativa leaves powder

Filtration

After maceration extract was filtered through whatman filter paper in a conical flask.

Figure Filtration of Cannabis Sativa extract

Rotary Evaporation

Filtered plant material was evaporated through rotary evaporator. Temperature of rotary evaporator was fixed according to the boiling temperature of each solvent.

Rotary evaporation

Screening of secondary metabolites from *Cannabis sativa*

Phenols

Phenols are widely distributed group of plant secondary metabolites.Presence of phenol change the color of extract into bluish black (as shown in Figure .1). Both ethanolic and methanolic extract showed strong positive results for all localities. On the other hand, variation was observed based on locality in the aqueous extract. Among all aqueous extracts, extracts from Multan has more phenols than extracts from Islamabad and Fateh Jang

Table 4.1: Qualitative analysis of phenols in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	+	+	++
Ethanol	+++	+++	+++
Methanol	+++	+++	+++

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

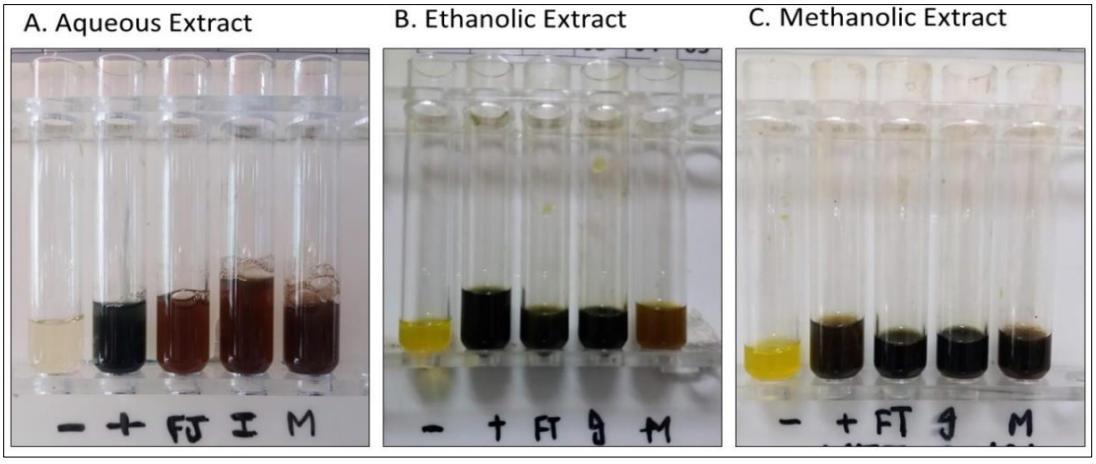


Figure 4.1: Test for detecting the presence of phenols in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

‘-’ representing negative control; ‘+’ representing positive control; ‘FJ’ representing Fateh Jhang; ‘I’ representing Islamabad and ‘M’ representing Multan

Alkaloids

Alkaloids are naturally occurring organic compounds also observed in *cannabis sativa* ethanolic, Methanolic and aqueous extract

Table 4.2: Qualitative analysis of Alkaloids in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	+++	+++	++
Ethanol	++	++	++
Methanol	+++	+++	+++

‘-’ = not present; ‘+’ = weakly present; ‘++’ = Moderately present ; ‘**+++’** = Strongly Present

3.4.2.

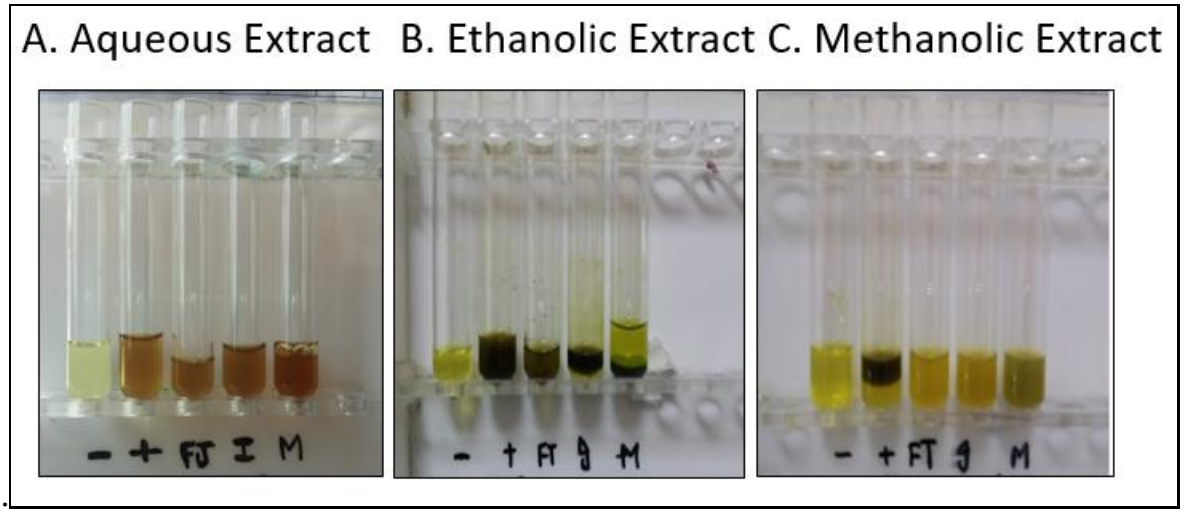


Figure 4.2.: Test for detecting the presence of alkaloids in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

‘-’ representing negative control; ‘+’ representing positive control; ‘FJ’ representing Fateh Jang; ‘I’ representing Islamabad and ‘M’ representing Multan

Anthraquinones

Anthraquinones are organic compounds which are present in some plants. Strongly present in *Cannabis sativa* Ethanolic extract slightly present in aqueous extract from all three localities. Anthraquinones were not observed Fateh Jang aqueous extract (shown in table 4.3). Color of the extract were turned into reddish brown (fig 4.3)

Table 4.3: Qualitative analysis of phenols in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jhang	**Islamabad**	**Multan**
Aqueous	+	+	++
Ethanol	+++	+++	+++
Methanol	-	-	-

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

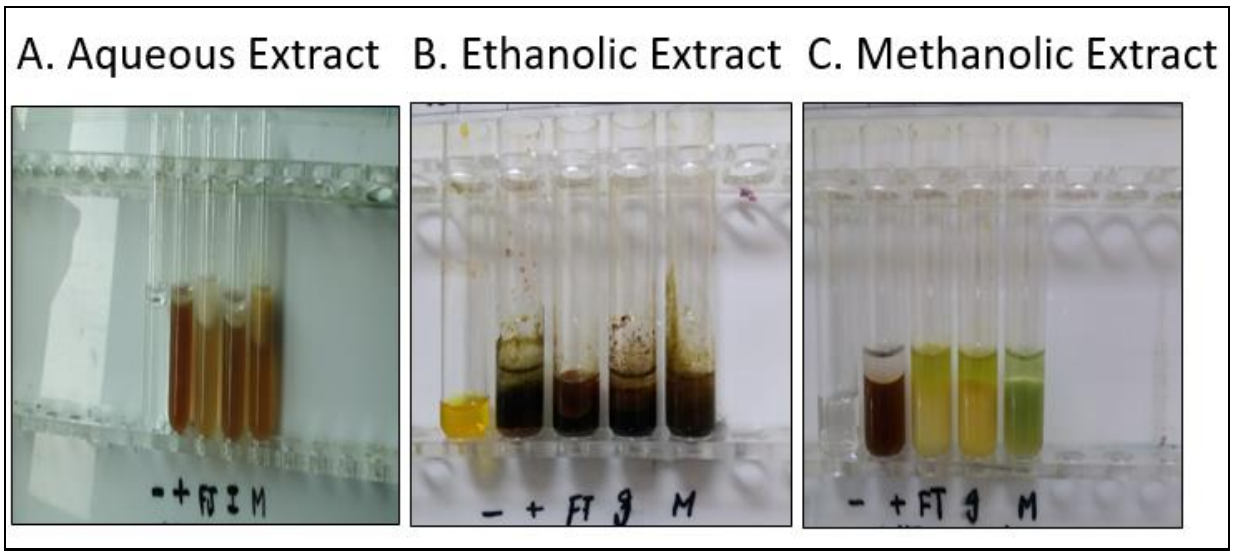


Figure 4.3: Test for detecting the presence of phenols in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jhang; 'I' representing Islamabad and 'M' representing Multan

Flavonoids

These are metabolites which are involved in the cell signaling pathways and mandatory for metabolism. Observed in all three types of ethanolic, methanolic and aqueous extract shown below (table 4.4) after performing the test yellow precipitates were formed in extract. All three extract have same results variation were not observed.

Table 4.4: Qualitative analysis of flavonoids in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jhang	**Islamabad**	**Multan**
Aqueous	+++	+++	+++
Ethanol	+++	+++	+++
Methanol	+++	+++	+++

'-' = not present; '+' = weakly present; '++' = Moderately present ; '**+++**' = Strongly Present

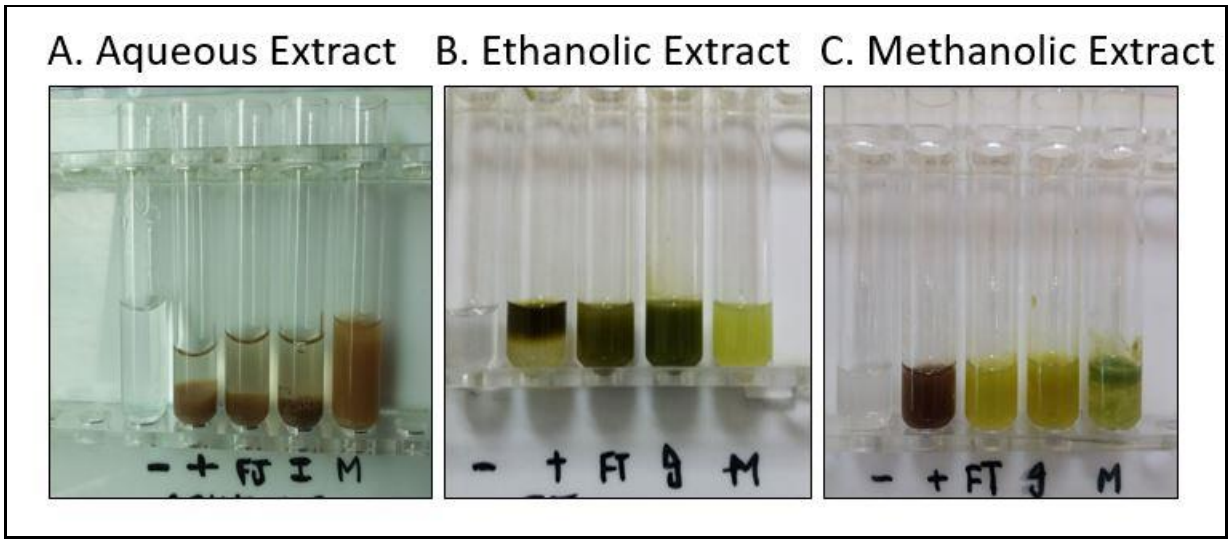


Figure 4.3: Test for detecting the presence of flavonoids in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Anthocyanin

The basic function of these pigments is to give red and purple colors to plants. Hence, they play a major role in providing color to plants. All three type of extracts lack anthocyanin because *Cannabis sativa* green in color there is no red or green color in plant. That's why test for anthocyanin have negative results. (Table 4.5)

Table 4.5: Qualitative analysis of Anthocyanin in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	Locations		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	-	-	-
Ethanol	-	-	-
Methanol	-	-	-

'-' = not present; '+' = weakly present; '++' = Moderately present ; '**+++**' = Strongly Present

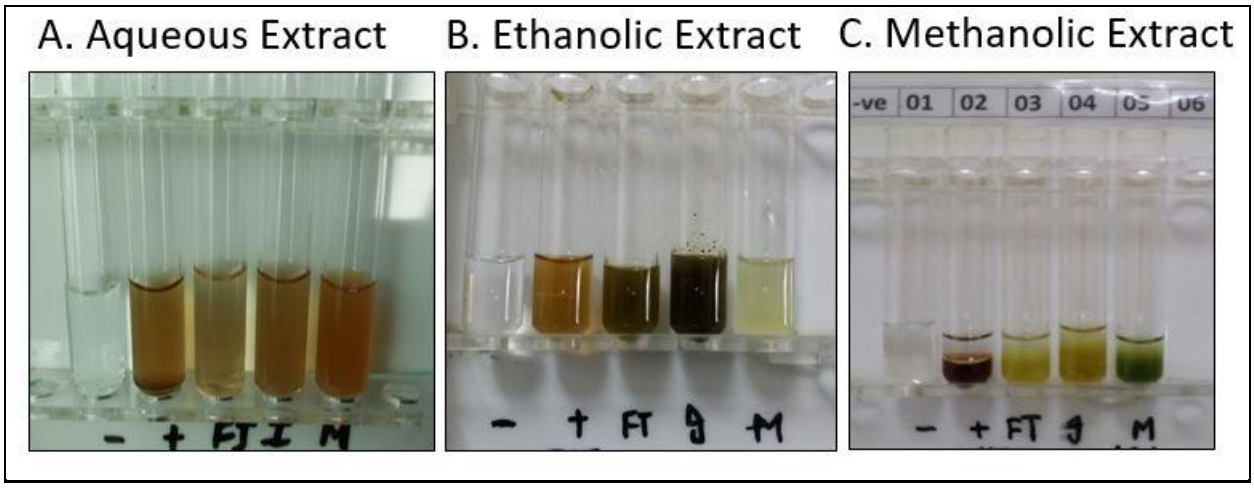


Figure 4.5: Test for detecting the presence of anthocyanin in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Leucoanthocyanins

These are the pigments related to anthocyanins. Presence of leucoanthocyanins change the organic layer of extract into red. Aqueous extract of *Cannabis sativa* from all three localities have leucoanthocyanin, ethanolic extract lack leucoanthocyanin and methanolic extract have moderate results. Which is depicted in (table 4.6 and figure 4.6)

Table 4.6: Qualitative analysis of leucoanthocyanin in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	+++	+++	+++
Ethanol	-	-	-
Methanol	++	++	++

'-' = not present; '+' = weakly present; '++' = Moderately present ; '**+++**' = Strongly Present

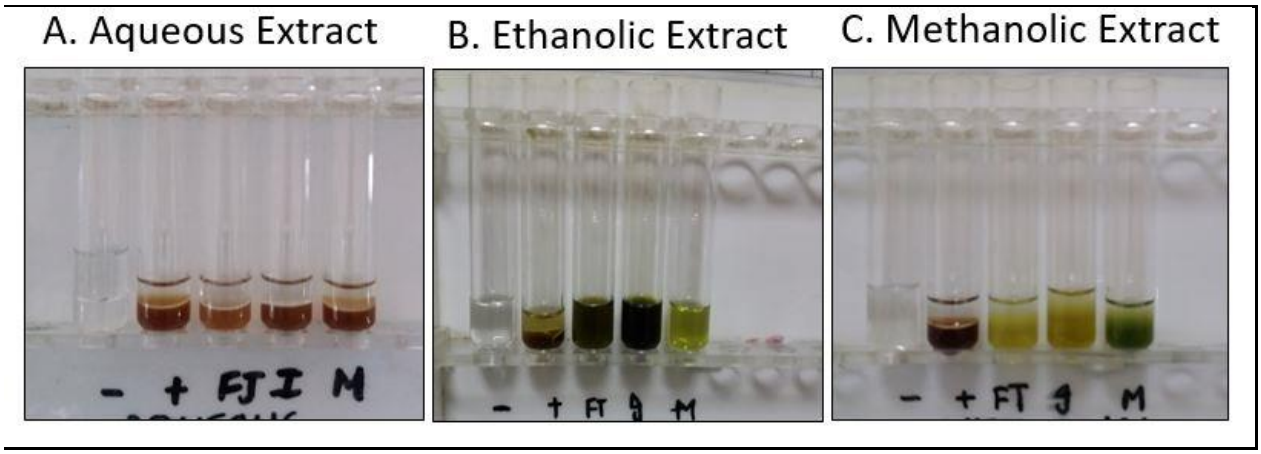


Figure 4.6: Test for detecting the presence of Leucoanthocyanins in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Tannins

Tannins are polyphenols present in plants and have pharmaceutical activities. All the three extracts of cannabis sativa showed strongly positive results for tannins. Aqueous extract of *Cannabis sativa* from Multan and Islamabad localities have strong results for tannins and Fateh jang extract have moderate results, while ethanolic extract has strong ratio in Fateh Jang and Islamabad and less in Multan. The methanolic extract on the other hand had strong results for all the three localities, as shown in the table below. (4.7)Extract gives transient greenish to black coloration as shown below (Figure 4.7)

Table 4.7: Qualitative analysis of tannins in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	++	+++	+++
Ethanol	++	+++	-
Methanol	++	++	++

'-' = not present; '+' = weakly present; '++' = Moderately present ; '**+++**' = Strongly Present

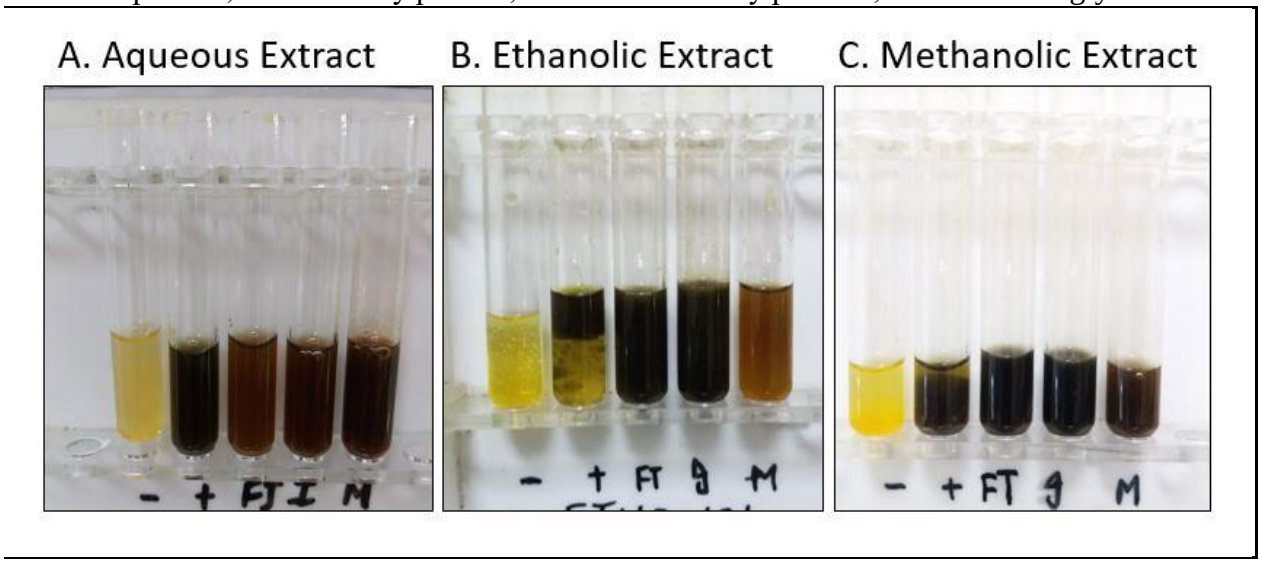


Figure 4.6: Test for detecting the presence of tannins in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Phlobatannins

Precipitates test was performed to observe the phlobatannins in *Cannabis sativa* extracts. The results showed that Multan locality of *Cannabis sativa* had strong aqueous extract, mild methanolic and not present in ethanolic extract. The observations showed that Islamabad locality sample had strong results for all the three extracts. The Fateh Jang locality showed strongest results of ethanolic extract and mild presence of methanolic extract, while the aqueous extract lack this metabolite. The variation of phlobatannins in the three extracts is due to different locality. The results are shown in the figure 4.8 below.

Table 4.8: Qualitative analysis of phlobatannins in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	-	+++	+++
Ethanol	-	+++	-
Methanol	++	+++	++

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

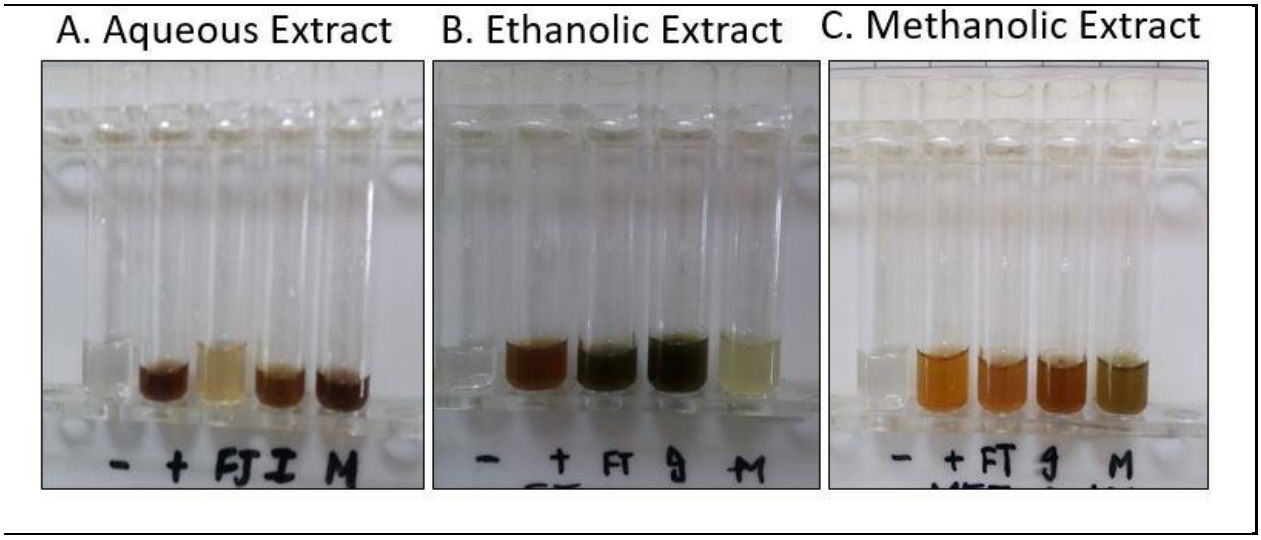


Figure 4.8: Test for detecting the presence of phlobatannins in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Coumarins

Coumarins are phytotoxic compounds, test were performed to check the presence of Coumarins in *Cannabis sativa* leaves extracts All three extracts from Fateh Jang sample have positive results. Aqueous extract and methanolic extract of Islamabad sample have moderate results while Ethanolic extract of Islamabad locality showed negative results for coumarins, aqueous, Ethanolic and methanolic extract of Multan sample have strong positive negative and moderate results respectively (table4.9). Extracts having coumarins turned into yellow color shown in figure 4.9.

Table 4.9: Qualitative analysis of coumarins in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	+++	+++	+++
Ethanol	++	-	-
Methanol	+++	++	++

'-' = not present; '+' = weakly present; '++' = Moderately present ; '**+++**' = Strongly Present

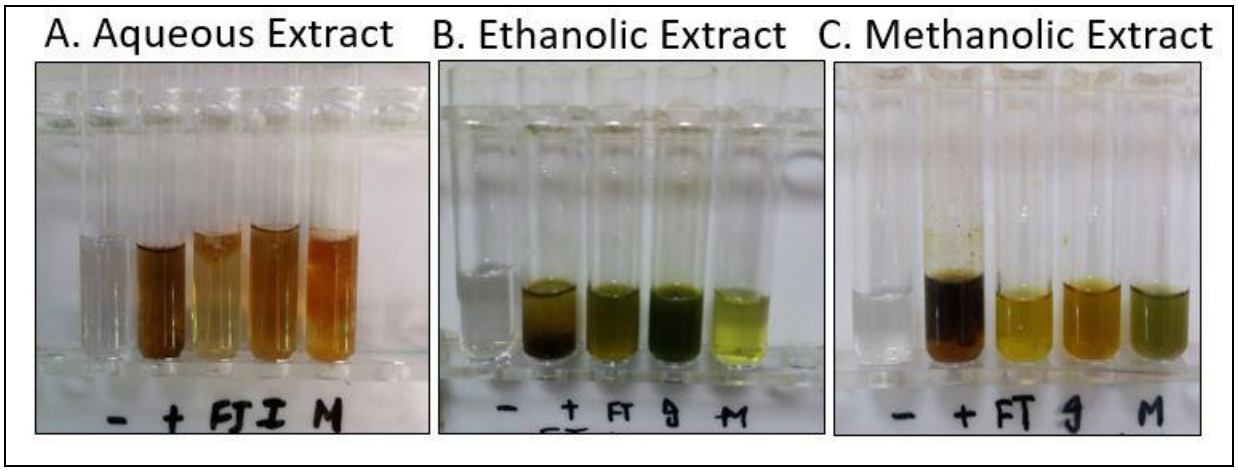


Figure 4.9: Test for detecting the presence of coumarins in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Terpenoids

Terpenoids metabolites stimulate development and growth and protect from biotic and abiotic environments in plants. They also play a major role in pharmaceutical industry. Test was performed to check the Terpenoids in all three types of extracts. Extracts having Terpenoids change into deep red color. The figure below depicts that Ethanolic and Methanolic extracts of Fateh jang and Islamabad locality have changed into deep red color. This result depicts strong presence of Terpenoids in Fateh Jang and Islamabad and absence in Multan locality. The aqueous extract has moderate results in all three localities as shown below.

Table 4.10: Qualitative analysis of terpenoids in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	++	++	++
Ethanol	+++	+++	-
Methanol	+++	+++	-

'-' = not present; '+' = weakly present; '++' = Moderately present ; '**+++**' = Strongly Present

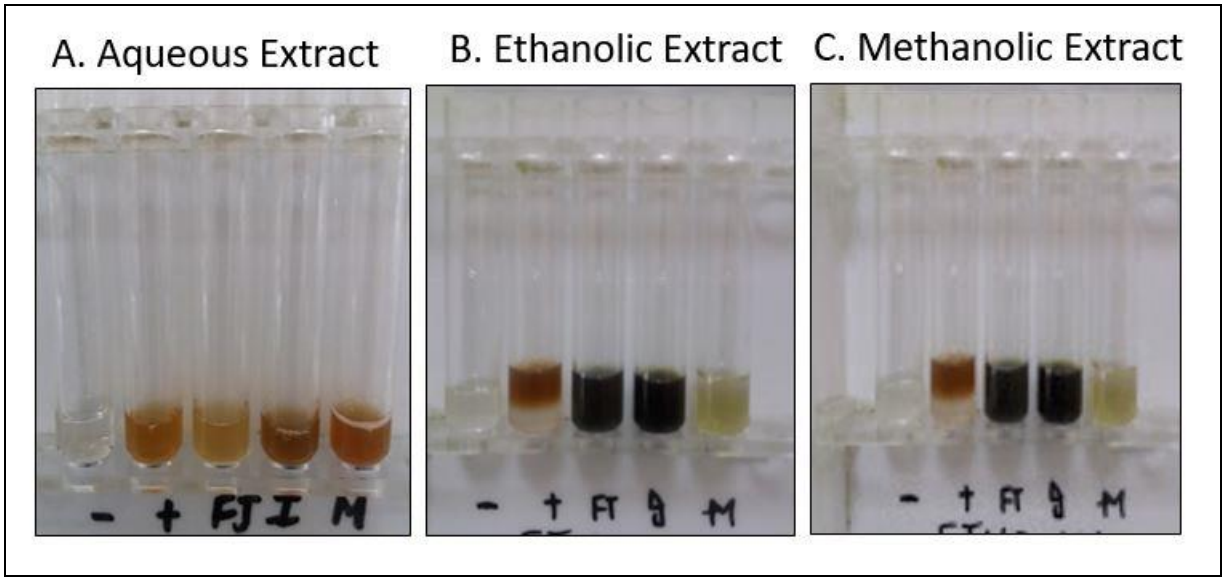


Figure 4.10: Test for detecting the presence of terpenoids in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Diterpenoids

These metabolites play major role in growth and development of plants and health care of humans. e.g. paclitaxel is used for treatment of cancer. *Cannabis sativa* leave extracts were treated chemically to observe presence of Diterpenoids in it. The results showed that all the extracts had presence of Diterpenoids which was confirmed due to the emerald green color of the extracts after the test.

Table 4.11: Qualitative analysis of diterpenoids in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	+++	+++	+++
Ethanol	+++	+++	+++
Methanol	+++	+++	+++

'-' = not present; '+' = weakly present; '++' = Moderately present ; '**+++**' = Strongly Present

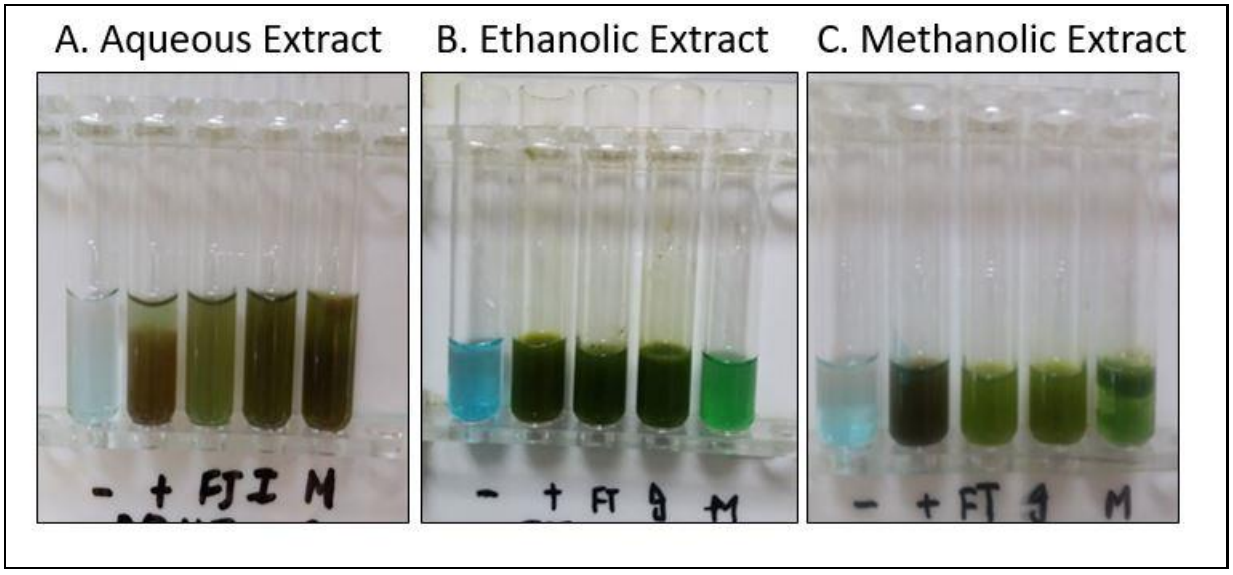


Figure 4.11: Test for detecting the presence of diterpenoids in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Steroids

Steroids are main secondary metabolites in plants. They have best anti-inflammatory properties due to which they are widely used in treatment of different diseases like arthritis and lupus. The extract of *Cannabis sativa* leave extract contains ample amount of steroids which was observed by performing Salkowski's test. Each of the extracts showed reddish brown color at the interface, which shows strong presence of steroids in the extract from all three localities.

Table 4.12: Qualitative analysis of steroids in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	+++	+++	+++
Ethanol	+++	+++	+++
Methanol	+++	+++	+++

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

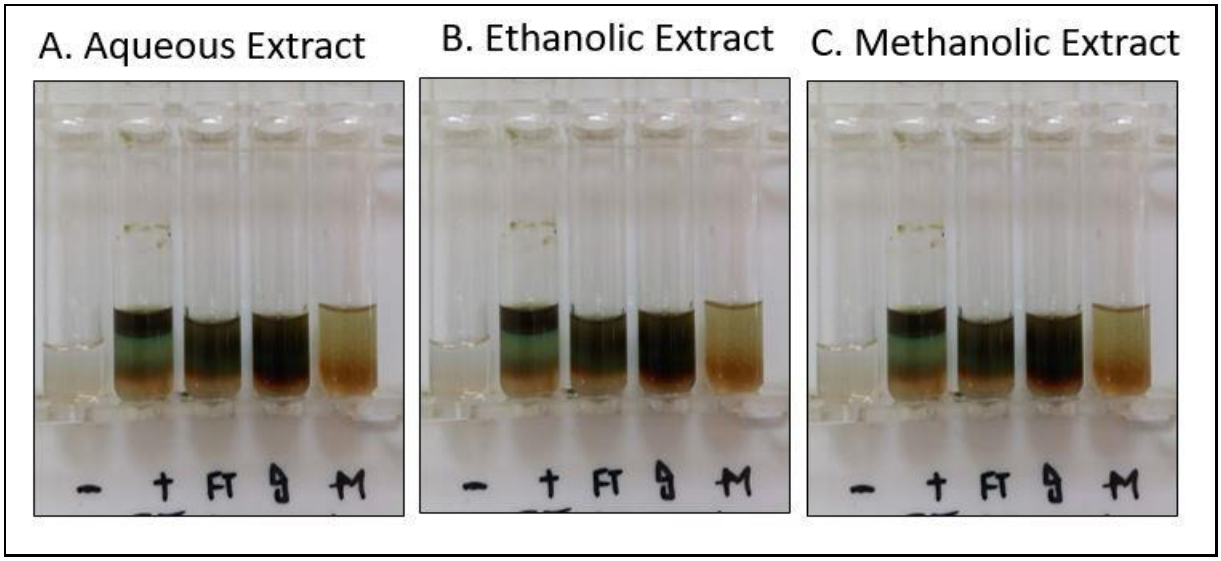


Figure 4.12: Test for detecting the presence of Steroids in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Sterols

Sterols of plants are known as phytosterols. They are helpful in control of cholesterol level in human body, hence control heart diseases. The Salkowski's test was performed on the extracts of *Cannabis sativa* from three samples. Ethanolic extract of all three localities have strong positive results as shown below (Table 4.13).The aqueous and Methanolic extract from Fateh Jang, Islamabad have moderate presence of sterols, while Multan has strongest result for sterols (Table 4.13). The appearance of red color at the lower layer indicates presence of steroids in *Cannabis sativa* extract (Fig 4.13).

Table 4.13: Qualitative analysis of sterols in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	++	++	+++
Ethanol	+++	+++	+++
Methanol	++	++	+++

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

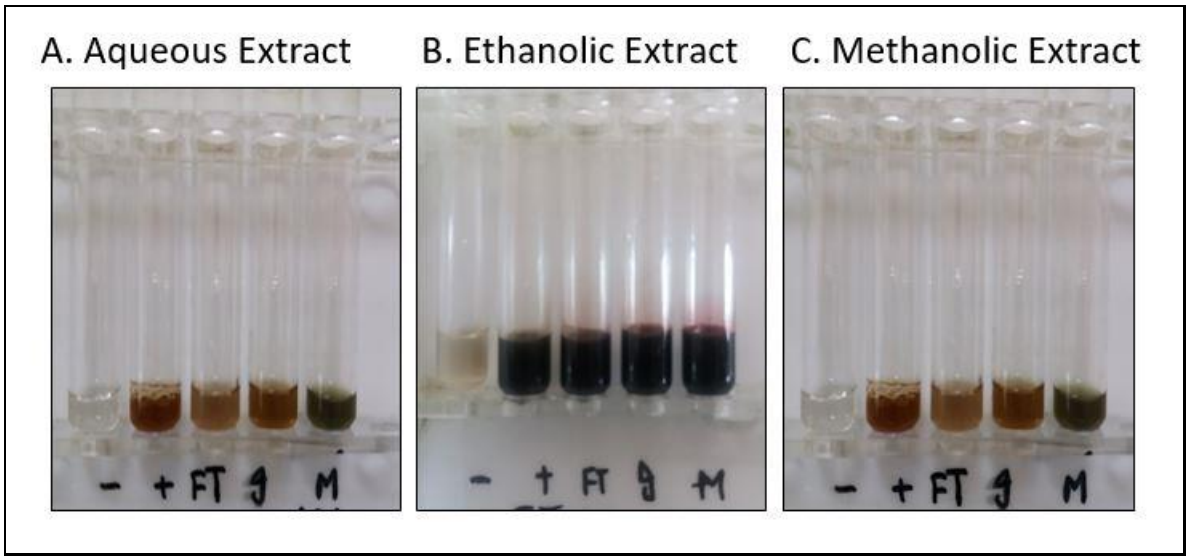


Figure 4.13: Test for detecting the presence of Sterols in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Saponins

Saponins are bioorganic molecules and aglycone. Foam test was performed to analyze the presence of Saponins in *Cannabis sativa* leaves extract. Ethanolic extract of Fateh jang and Islamabad have positive results, Multan sample have negative results. Methanolic extract from all three localities lack Saponins while aqueous extract from Multan and Islamabad sample have moderate results and Fateh jang sample shown the absence for Saponins(Table 4.14) Presence of Saponins gives froth appearance to extract after performing foam test depicted in (Figure 4.14).

Table 4.14: Qualitative analysis of Saponins in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	++	++	-
Ethanol	++	+++	-
Methanol	-	+++	-

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

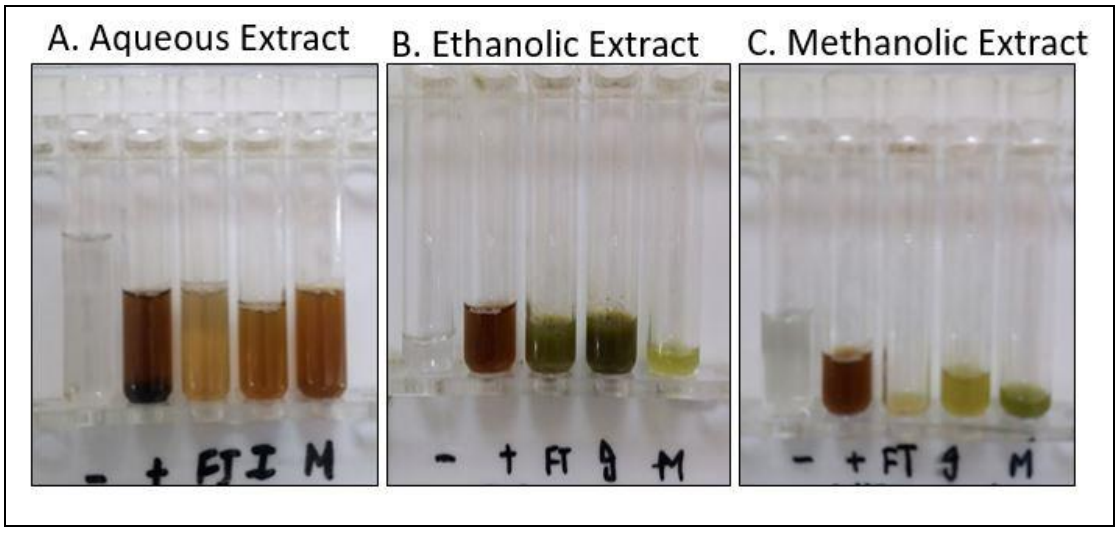


Figure 4.14: Test for detecting the presence of Saponins in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Resins

In folk as well as modern world's medicines resins used due to its bioactive and pharmaceutical properties. Phytochemical screening of *Cannabis sativa* leaf extract for resins have variation in results due to difference in localities (shown in table 4.15) Methanolic and ethanolic extract of all three localities sample have strong positive results for resins while aqueous extract lack resins below in (Figure 4.15)

Table 4.15: Qualitative analysis of Resins in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	-	+++	+++
Ethanol	+++	+++	+++
Methanol	-	+++	+++

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

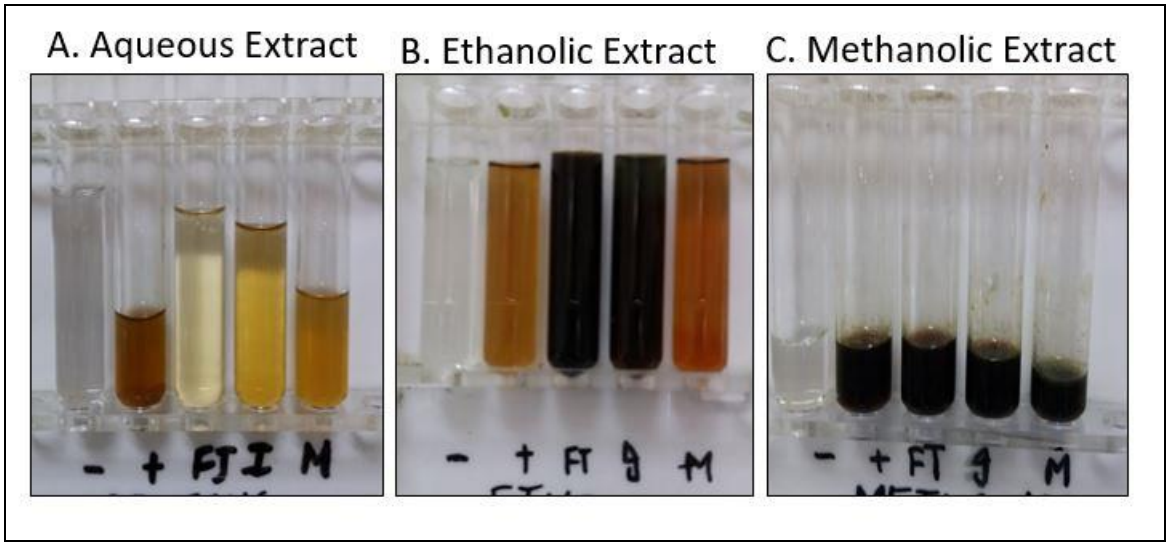


Figure 4.15: Test for detecting the presence of Resins in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Glycosides

These are the secondary metabolites which are used as medicine in therapeutics. The results varied among all the three extracts of different localities. The aqueous and Methanolic extract from Islamabad and Fateh Jang showed moderate results, while Multan had strong presence of glycosides. The Ethanolic extract of Fate Jang and Islamabad showed violet and blue coloration which shows strong results as shown in figure below (Figure 4.16). Multan had moderate results for the Ethanolic extract amongst all other extracts.

Table 4.16: Qualitative analysis of Glycosides in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	++	++	+++
Ethanol	++	++	+++
Methanol	+++	+++	++

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

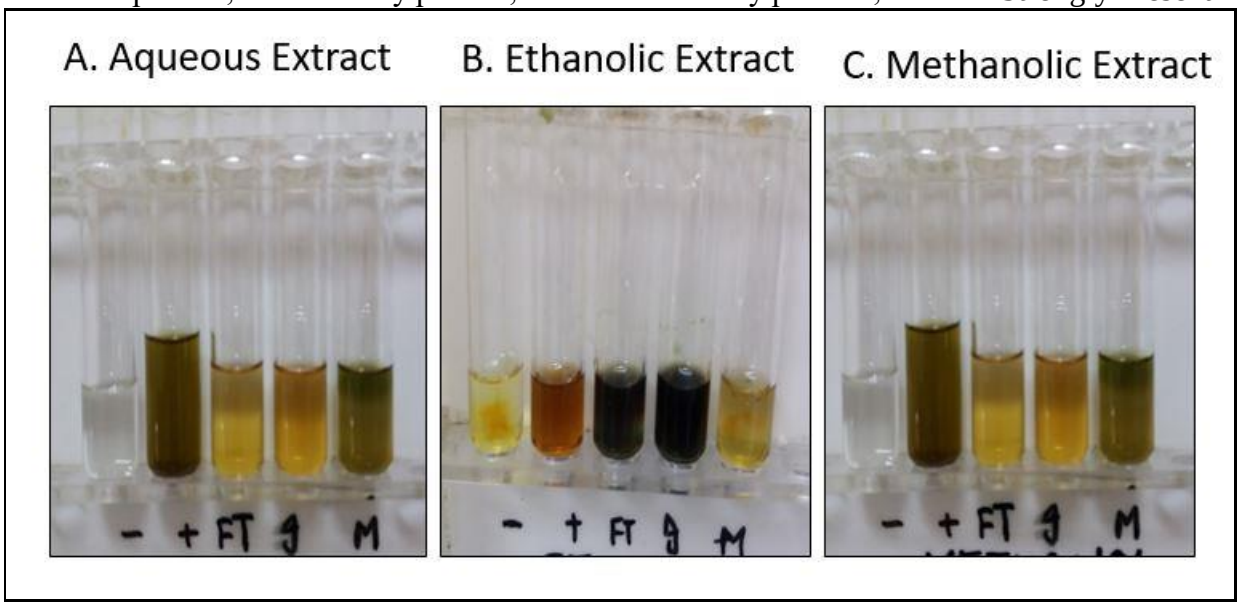


Figure 4.16: Test for detecting the presence of Glycosides in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Emodins

These are the secondary metabolites which have anti-inflammatory and anti-oxidant effects.The extracts having emodins change into red color after the test. The results showed that all the three extracts prepared from sample of three localities lack emodins (Table 4.17).

Table 4.17: Qualitative analysis of Emodins in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	Locations		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	-	-	-
Ethanol	-	-	-
Methanol	-	-	-

'-' = not present; '+' = weakly present; '++' = Moderately present ; '**+++**' = Strongly Present

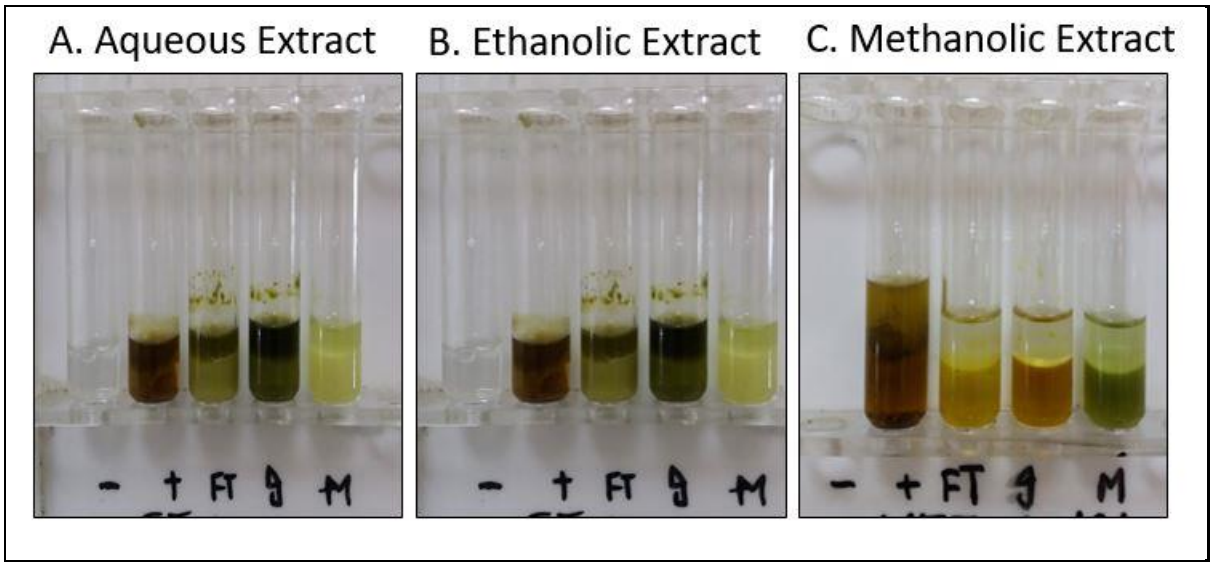


Figure 4.17: Test for detecting the presence of Emodins in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Amino acids

These are the most important metabolites, involve in the maintenance of body function. Ninhydrins test was performed to check the presence of amino acids in *Cannabis sativa.* The violet color of the extract as shown in the fig.4.18 shows strong presence of amino acids. The results of all the three extracts were strongly positive except Ethanolic extract from Multan sample which showed moderate results. (Table 4.18).

Table 4.18: Qualitative analysis of Amino acids in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	Locations		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	+++	+++	+++
Ethanol	+++	+++	++
Methanol	+++	+++	+++

'-' = not present; '+' = weakly present; '++' = Moderately present ; '**+++**' = Strongly Present

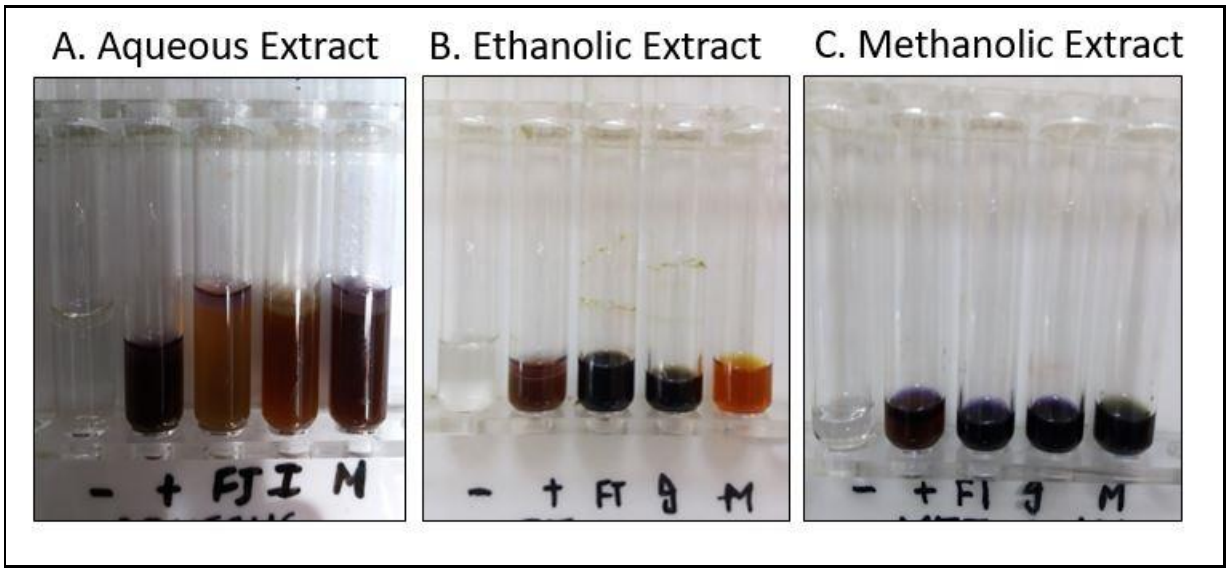


Figure 4.18: Test for detecting the presence of Amino acids in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan.

Proteins

Proteins are essential metabolites of the body xanthoproteic test was performed to check the presence of proteins, which involved boiling of the extract. The boiling process changed the precipitates into dark brown color which shows that protein present in the extract was de natured during the boiling procedure. The extracts from all the three localities had strong and moderate presence of proteins similar to amino acids.

Table 4.19: Qualitative analysis of Proteins in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	++	++	+++
Ethanol	+++	+++	+++
Methanol	++	+++	+++

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

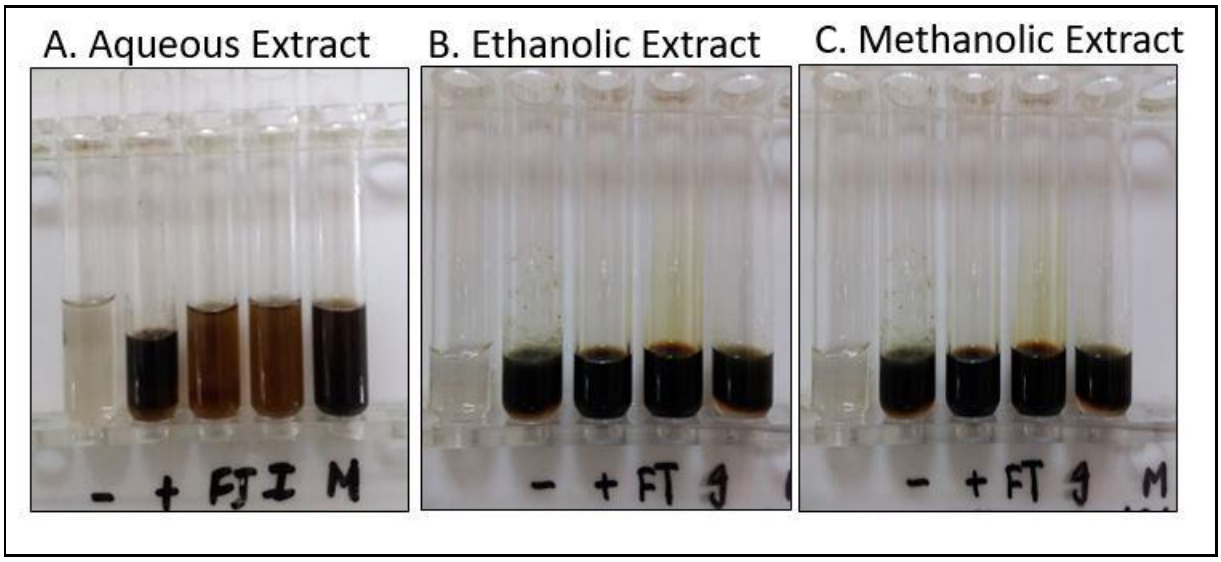


Figure 4.19: Test for detecting the presence of Proteins in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

Carbohydrates

Fehling's Test was performed for observing presence of carbohydrates in the extract of *Cannabis sativa* from Islamabad, Multan and Fateh Jang. All the extracts showed strong positive results for proteins except Ethanolic extract in Multan sample.

Table 4.20: Qualitative analysis of Carbohydrates in extracts prepared from *Cannabis sativa* L. collected from various localities

Extracts	**Locations**		
	Fateh Jang	**Islamabad**	**Multan**
Aqueous	+++	+++	+++
Ethanol	+++	+++	++
Methanol	+++	+++	+++

'-' = not present; '+' = weakly present; '++' = Moderately present ; **'+++'** = Strongly Present

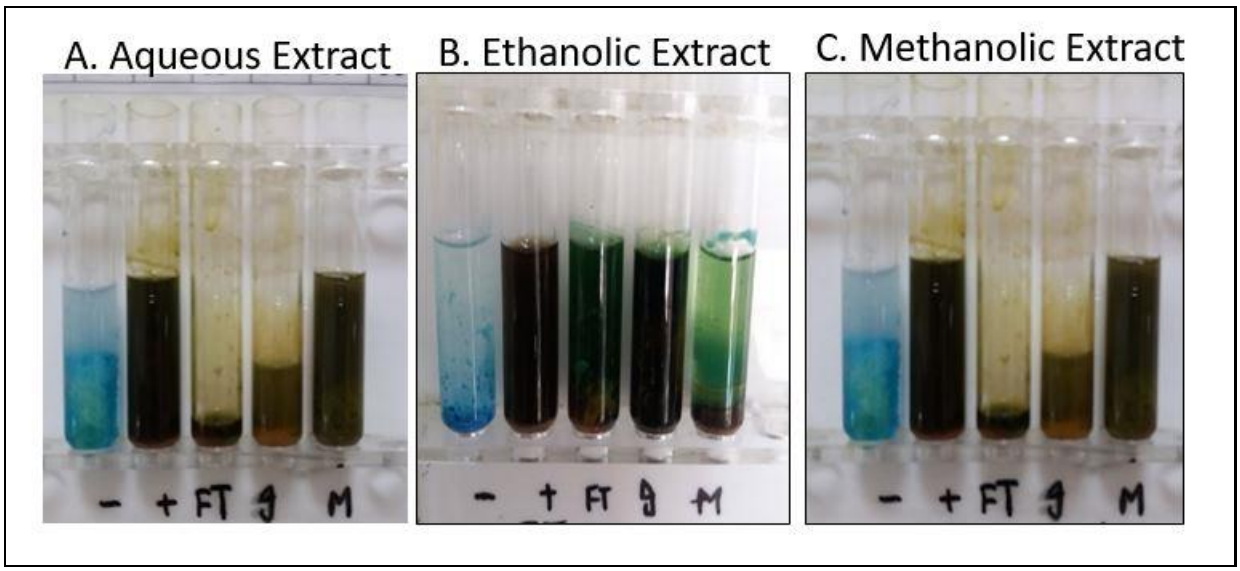


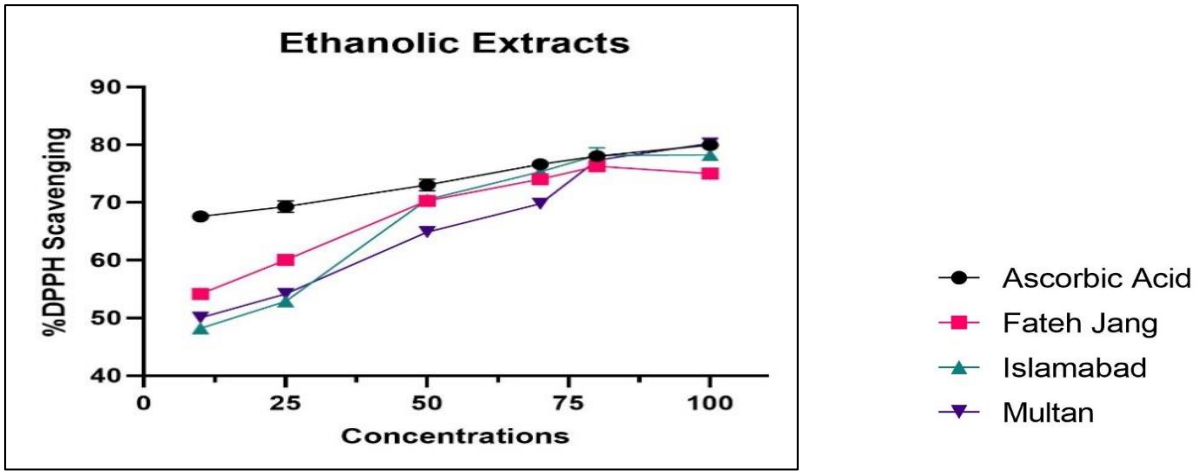
Figure 4.20: Test for detecting the presence of Carbohydrates in (A) Aqueous, (B) Ethanolic and (C) Methanolic extract of Cannabis sativa L. collected from various localities

'-' representing negative control; '+' representing positive control; 'FJ' representing Fateh Jang; 'I' representing Islamabad and 'M' representing Multan

DPPH Essay

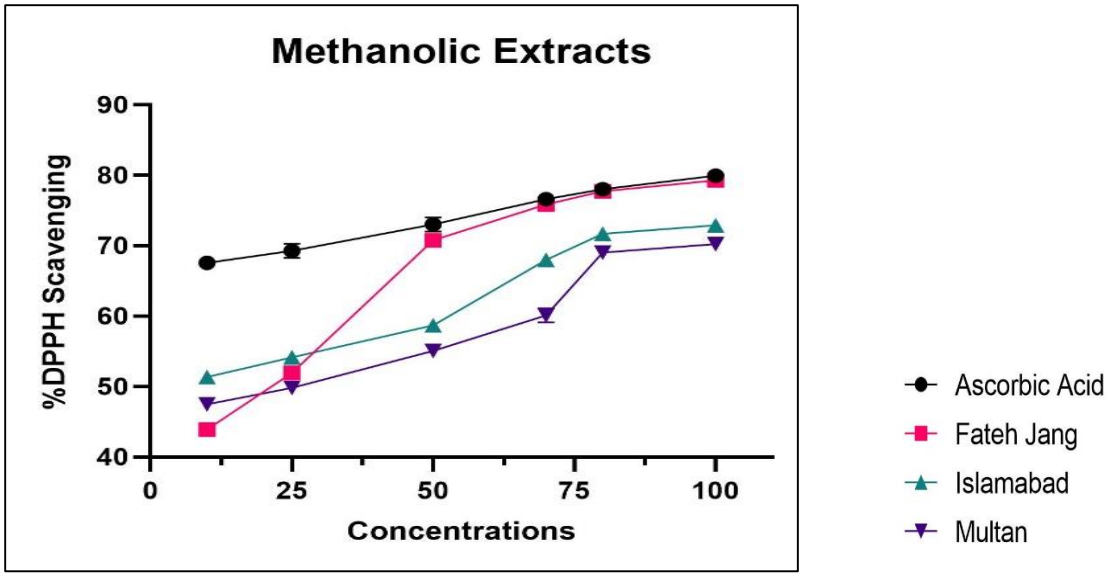
Free radical scavenging activity were performed to analyze the antioxidant potential of each extract of *Cannabis sativa* obtained from three different localities. *Cannabis sativa* sample were collected from Multan, Islamabad and Fateh Jang. Ethanolic, Methanolic and aqueous extracts were prepared from Ascorbic acid were used as standard antioxidant agent have maximum antioxidant potential here shown in graph. With increasing concentration of extract scavenging activity were also increased for each extract.

Graph 1 below depicting free radical scavenging activity of Ethanolic exract of all three localities. In Ethanolic extract Multan sample have maximum antioxidant activity as compared to Islamabad and Fateh Jang sample.



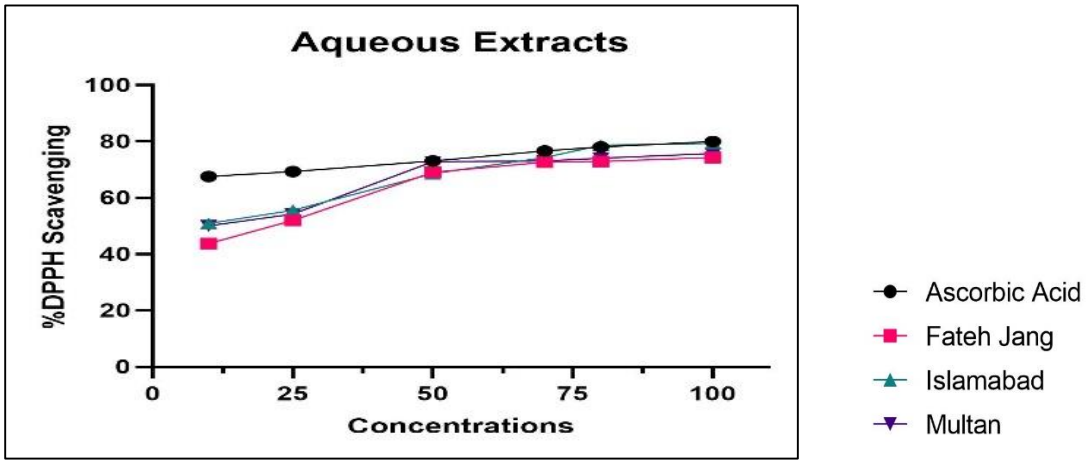
Graph 1

Graph 2 depicting results of free radical scavenging activity of Fateh Jang, molten and Islamabad Methanolic extracts. All three samples have increasing order, Fateh jang sample have maximum antioxidant potential as compared to Islamabad and Multan.



Graph 2

Graph 3 showing the antioxidant potential for aqueous extract prepared from Islamabad, Multan and Fateh jang *Cannabis sativa* leaf powder extract. All three types of extracts showing same antioxidant potential

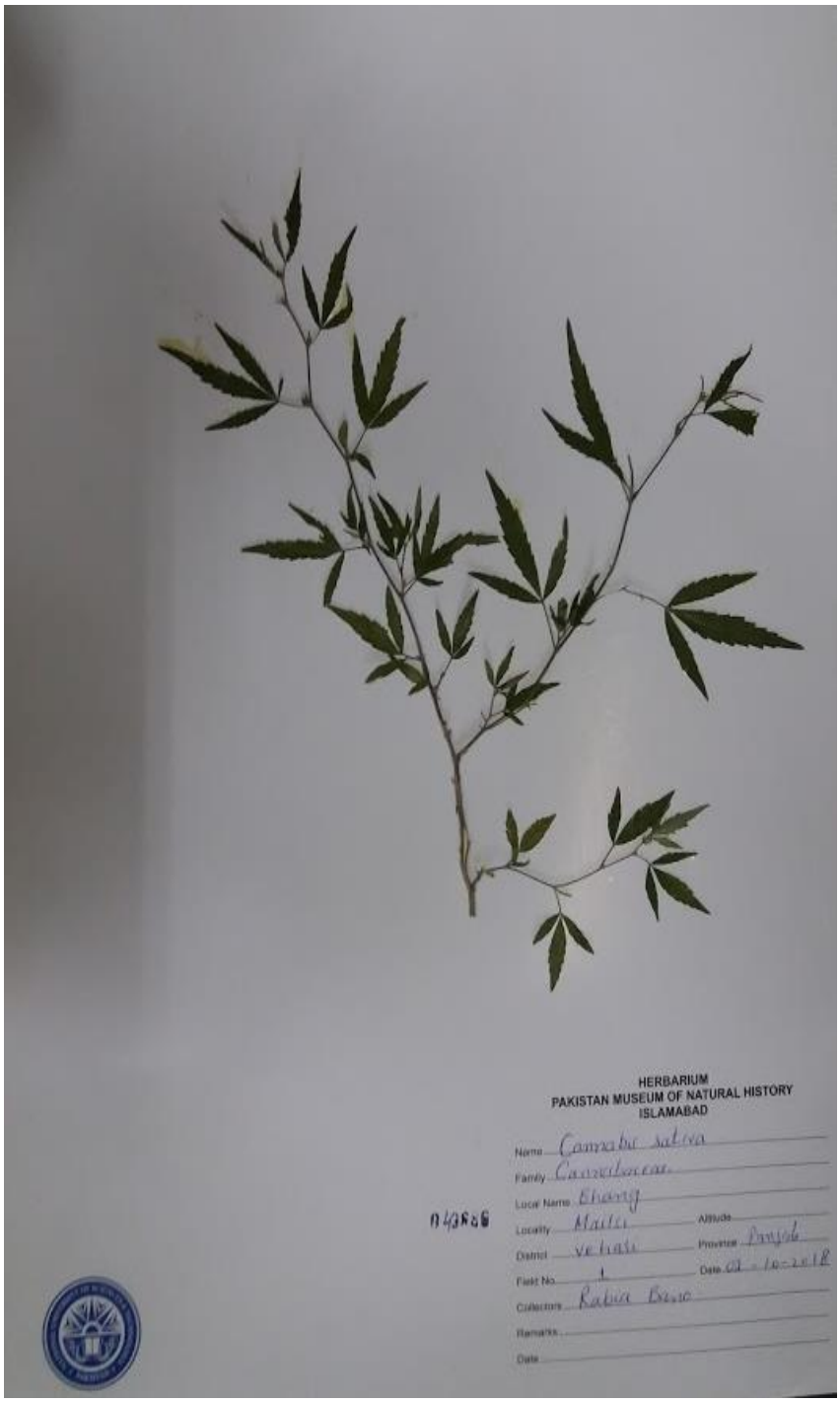


Graph 3

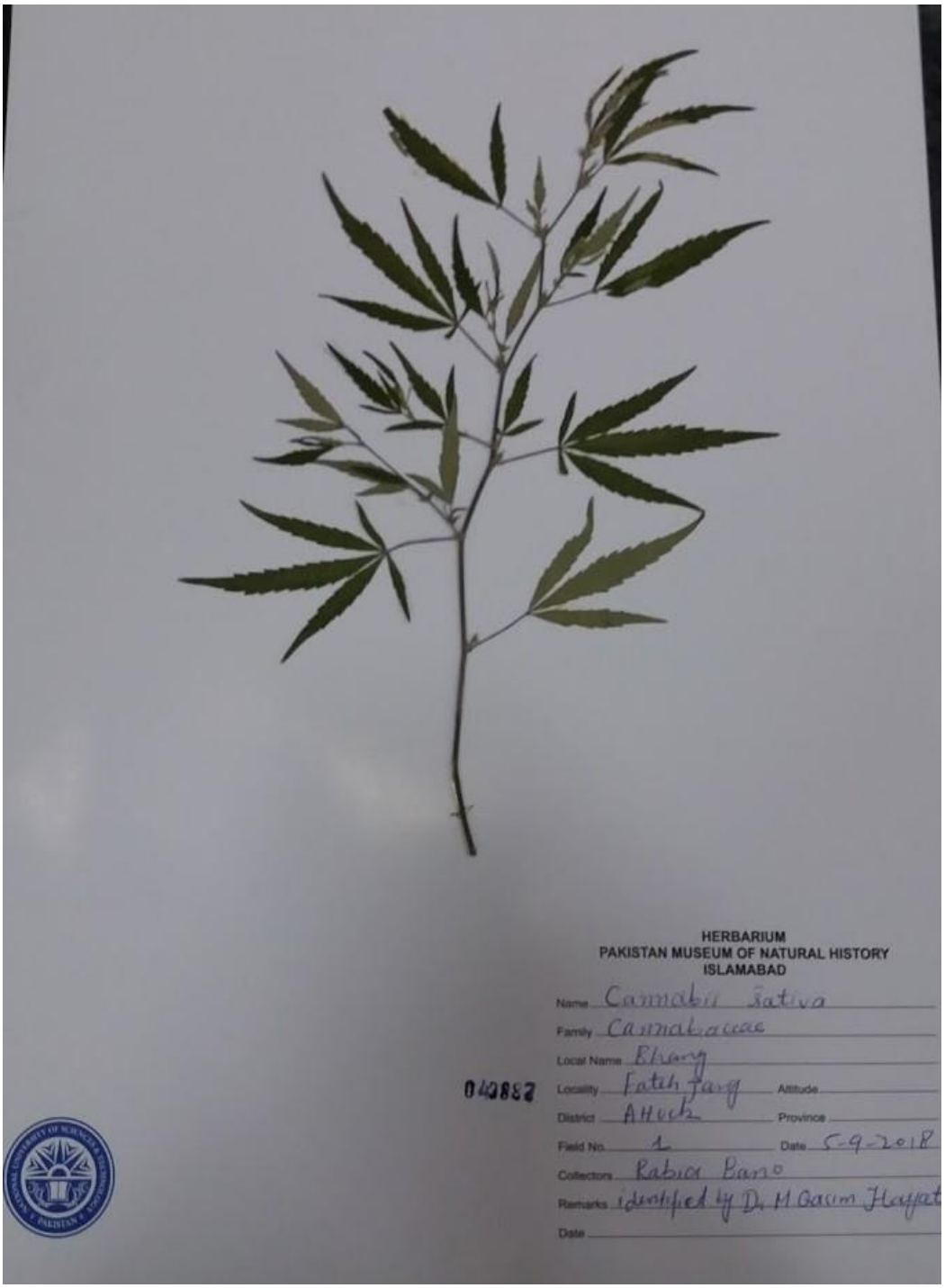
Identification of Plant

Herbarium specimen of *Cannabis sativa* submission

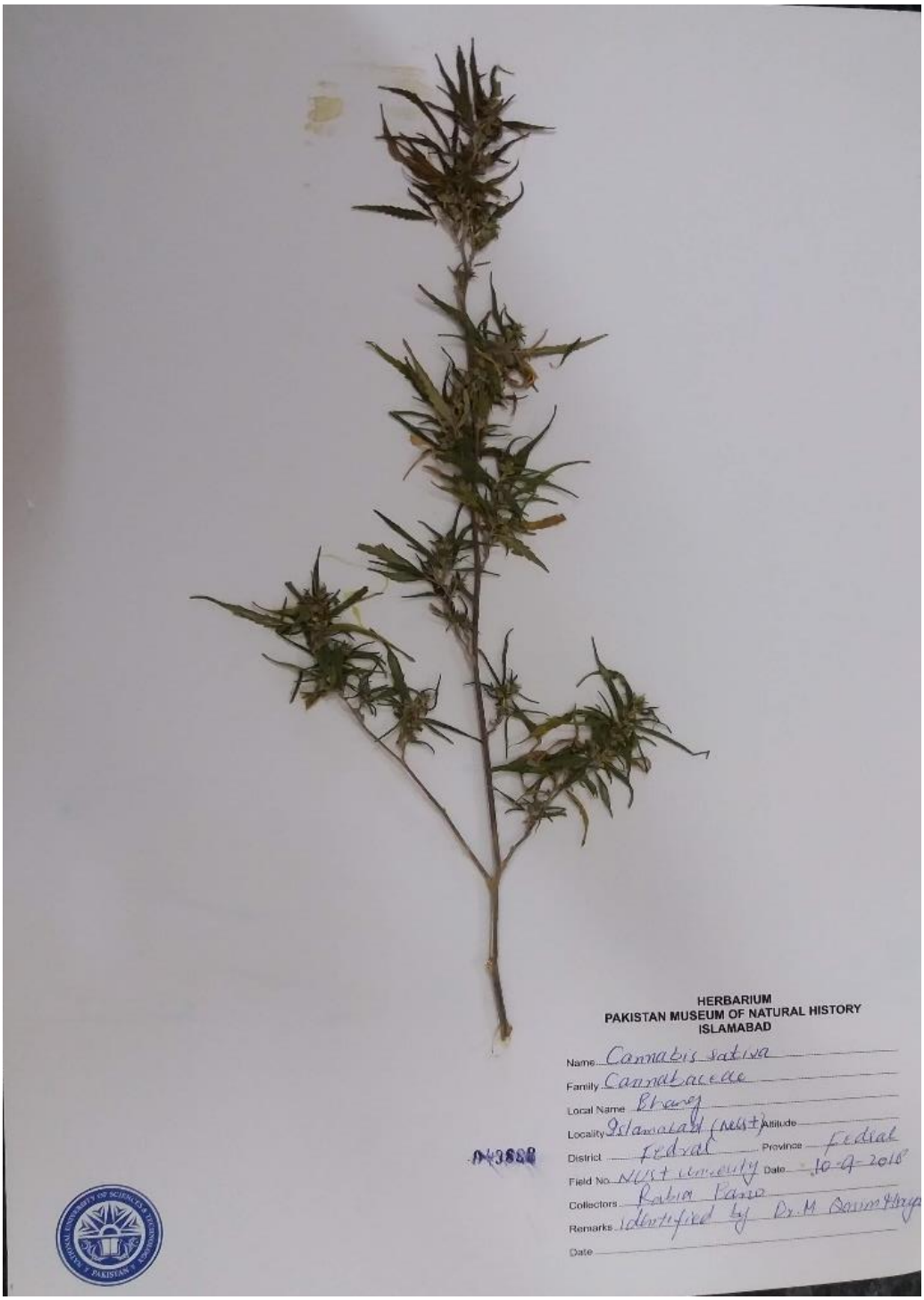
Herbarium specimen of Islamabad, Multan and Fateh jang sample were prepared and mounted on sheet and submitted to Pakistan Museum of Natural History and accession numbers were obtained for Multan (043886), Fateh Jang(043887) and Islamabad(043888).



Herbarium specimen from Multan sample of *Cnnnabis sativa*



Herbarium specimen from Fateh jang sample of cannabis sativa



Herbarium specimen from Islamabad sample of *Cannabis Sativa*

Barcoding of plant

For molecular identification DNA extraction was done through CTAB methods and ITS region was amplified using universal primers and approximately 750 bp amplicon was obtained further confirmation was done through sanger sequencing.

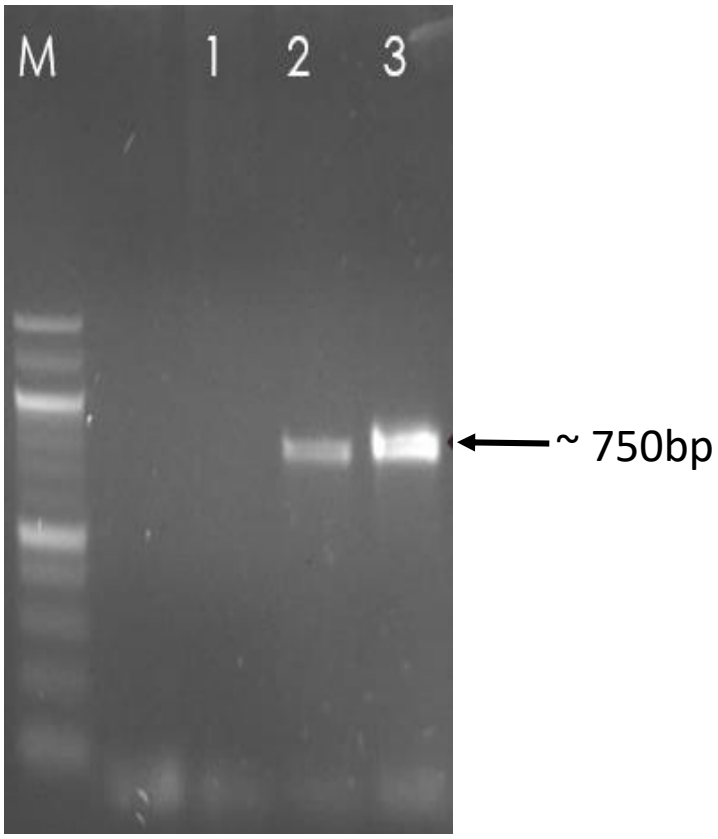


Figure: Gel electrophoresis of ITS Region based PCR amplification of Cannabis sativa, M= ladder [100bp], 1=negative control, 2=positive control and 3= ITS region amplification from Multan sample.

Phylogenetic study

Phylogenetic study of ITS region of *Cannabis sativa* were conducted using geneious software. (http://www.geneious.com,Kearse et al.,2012) ITS gene sequence of *Cannabis sativa* obtained from sequincing. Phylogenetic tree were constructed through Maximum Likelihood, Neighbour Joining and Bayesian methods. Trees constructed from three methods were same which confirmed phylogeneticly, specie is *Cannabis sativa.*

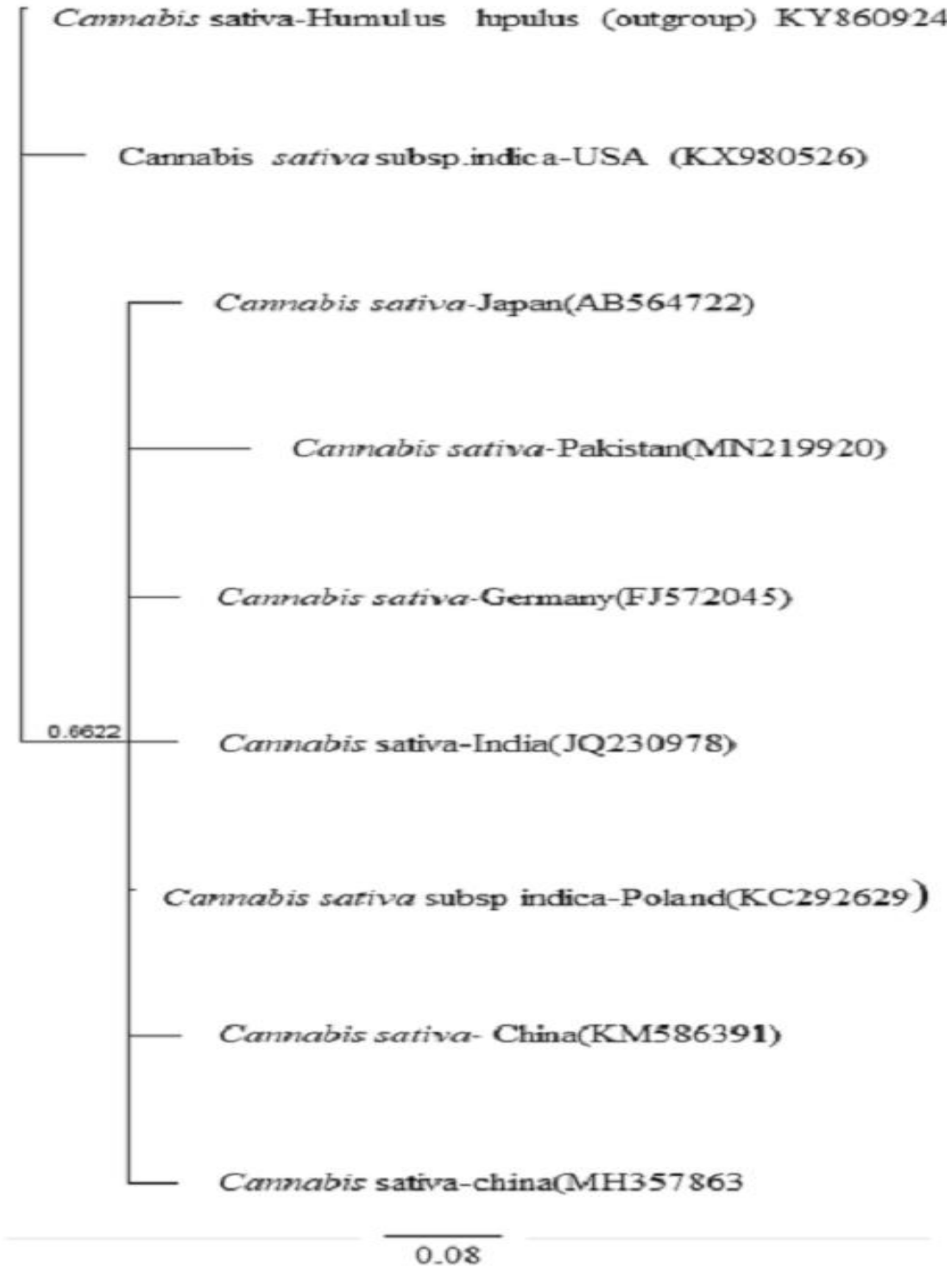


Figure: Bayesian tree of Cannabis sativa based on ITS region

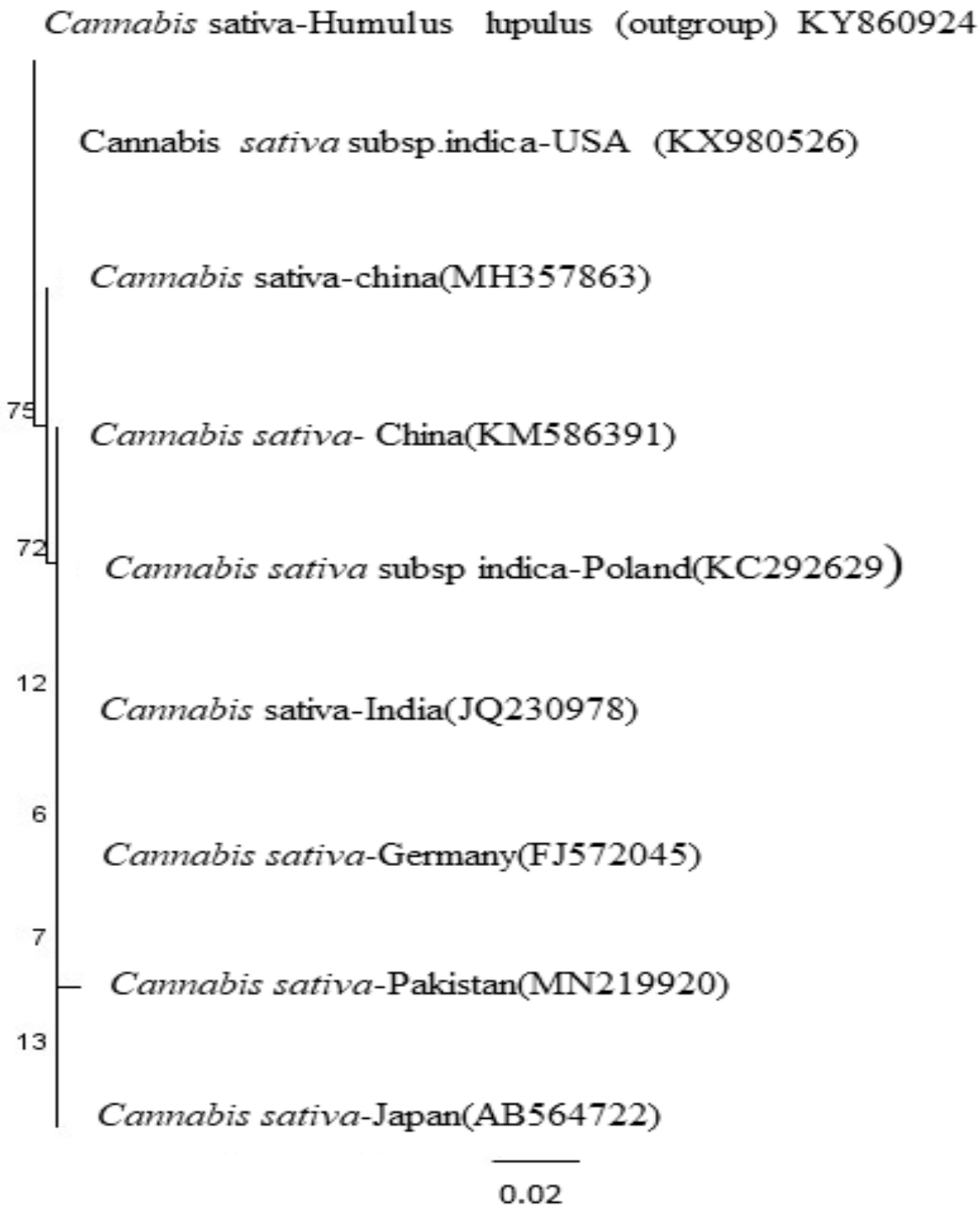


Figure:Maximum likelihood tree of Cannabis sativa based on ITS region

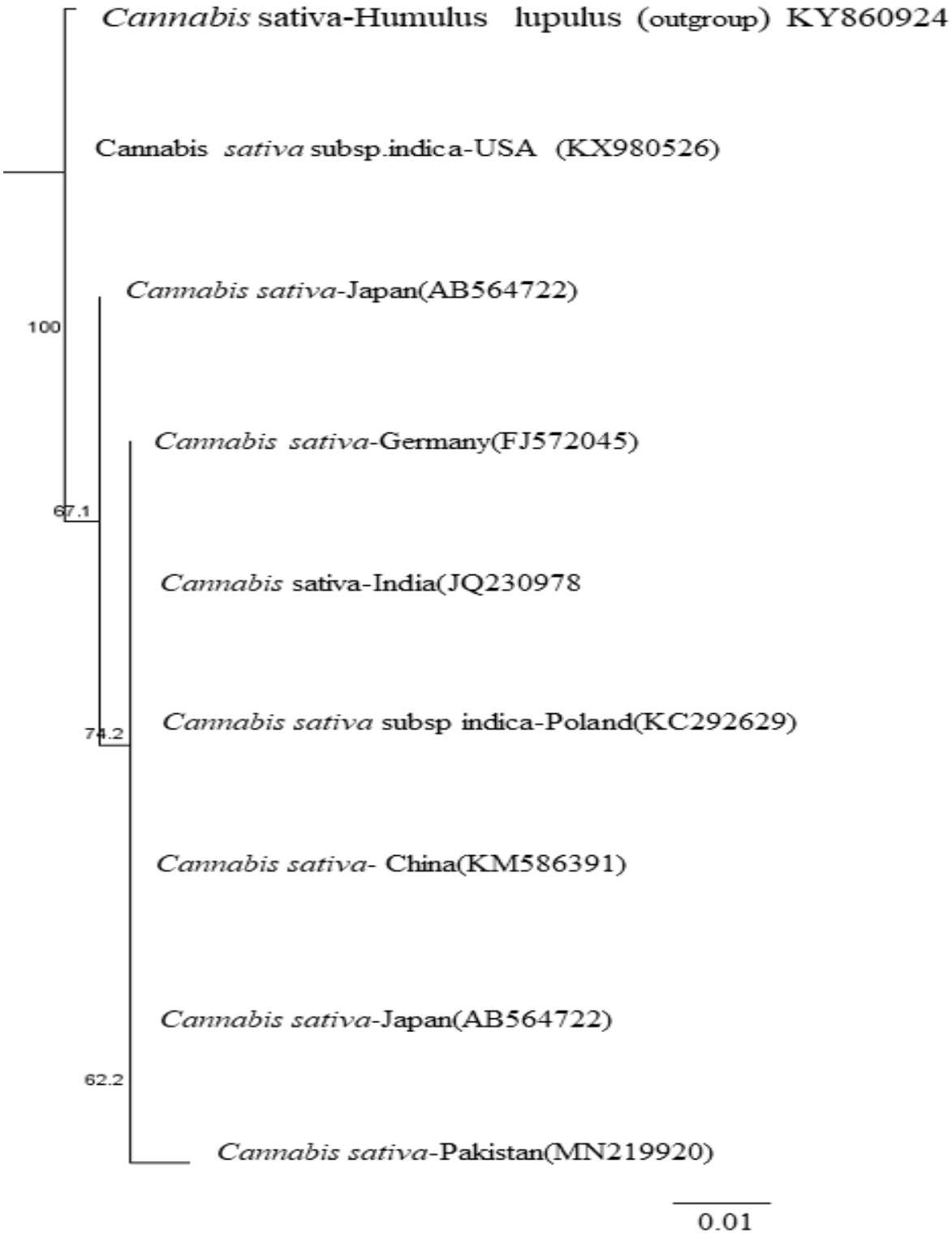


Figure: Neighbor joining tree of Cannabis sativa based on ITS region

Chapter no. 5

DISCUSSION

Discussion

Medicinal plants grow naturally in many countries of the world. They have gained global importance and are crucial for futher development in various scientific avenues. A Combination of all the research conducted by a group of scientists aids in determining whether a medicinal plant is suitable for making medicines or not. *Cannabis* is a genus containing medicinal plants which are used due to their medicinal value all over the world since ancient times. The first species of *Cannabis* to be classified is *Cannabis sativa*. It is a rich source of naturally occurring compounds. *Cannabis sativa* now attracted a wave of research attention as an herbal medicine. Scientists assume that Secondary metabolites mostly produced by members of the *Cannabis* genus. In the present study focus was narrowed down to screen these secondary metabolites from *Cannabis sativa,* secondly to measure the antioxidant activity of *Cannabis sativa* leaf extracts and lastly to identify *Cannabis sativa* through amplification and sequencing of ITS region. This study developed an understanding towards natural phytochemicals present in *Cannabis sativa. Cannabis sativa* has mixtures of phytochemicals, such as phenolic compounds, glycosides, alkaloids, flavonoids, proteins, amino acids, emodins and carbohydrates. The *Cannabis sativa* samples were collected from Islamabad, Multan and Fateh Jang. Three types of Extracts were prepared from leaf powder and analyzed phytochemically. The phytochemical analysis of the same species of *Cannabis sativa* from three different localities varied in phytochemical profile.

Methanolic extract from Islamabad, Multan, and Fateh Jang sample have different type of phytochemicals. Carbohydrates, proteins, amino acids and anthraquinones were present in all three locality extracts. Resins were not present in Fateh Jang sample while it was present in Islamabad and Multan sample. The results of the extract prepared in methanol were relevant to the results presented by (Maqsood, et al., 2019), as anthocyanins and terpenoids were absent. The

Methanolic extract of Multan sample lacked phenolic compounds while Islamabad and Fateh Jang sample had phenols.Aqueous extract of all three localities contain alkaloids, phenols anthraquinones whereas the difference in the presence of saponins, terpenoids. The aqueous extracts of *Cannabis Sativa* revealed presence of alkaloids, tannins and saponins in a research study conducted in Nepal (Devkota, Sharma, Raj, & Jha, 2013).

Ethanolic extract also have phytochemical difference in all three localities. Alkaloids, flavonoids, carbohydrates, phenols, saponins, terpenoids, proteins, glycosides were present in the experiments conducted by (Ullah, et al., 2018) at a sample of Bajur Agency, which is relatively a colder climate as compared to Multan and Fateh Jang. The extract of *Cannabis sativa* from Multan prepared in ethanol lacked emodins, terpenoids, phlobatannins, coumarins, tannins and leucoanthocyanins unlike the study conducted by (Ullah, et al., 2018). While the results from Islamabad and Fateh Jang ethanolic extracts were consistent with the study conducted on the sample from Bajur Agency. Difference might be due to the climate change among all the localities. Metabolites also depends on properties of solvents used for extract making.

Antioxidants are the modulators of cell signaling processes. Results obtained after performing DPPH essay showed that *Cannabis sativa* have high antioxidant power at different concentration of extracts. Of all the three extracts distilled water extracts showed a stable performance and the curve was stable in comparison to methanolic and ethanolic extracts. The lowest value on the graph was by 10% conc. Methanolic extract of Fateh jang at 43% scavenging activity. While the maximum activity was by 100% conc. Methanolic extract of Fateh jang at 78% scavenging activity. The graph of Methanolic and ethanolic extracts was curvy which implies variation in activity from low to high concentration. The curve of Methanolic extracts of Multan shows the lowest values implying that this extracts shows the least antioxidant activity in comparison to all

the extracts. On the other hand ethanolic extracts of Fateh jang consistently show a high curve. This proves it to be the best antioxidant extract of all the extracts of this study. The differences in extracts may be due to various reasons yet cannabinoids being the unique secondary metabolite of cannabis need to be further evaluated. This leads to the fact that Cannabinoids most definitely improve signaling pathways and performance of enhancer and ligand. Another important aspect of this observation is the first line of defense to protect against cell degradation, disease and tumor formation is antioxidants. For the previous many years research has been conducted to discover compounds and synthesize drugs with multi-dimensional approach. Cannabis in recent years has gained attention and massive support for its economic benefits. This study was executed to compare the species growing in three distinct localities. Antioxidant potential of all specimens was proved which makes cannabis specimens of Pakistan a possible candidate for further pharmacological studies.

Chapter no. 6

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