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Pharmaceutical and Biomedical Characterization of Cannabinoids in *Cannabis sativa* L. using Gas Chromatography-Mass Spectrometry (GC-MS)

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Abstract

Cannabis is the most frequently used drug of abuse not only in Pakistan as well as the rest of the globe. Its use is increasing drastically every year. Every year, its use rises dramatically. *Cannabis sativa* can be analyzed using GC-MS to reveal the variations in the components of this plant harvested from Tirah valley. Cannabidiol (CBD), one of the biologically active substances found in naturally occurring cannabinoids, was found in significant concentrations in both ethanolic and *n*-hexane extracts in addition to other cannabinoids. Understanding the plant's components will help determine its prevalence. When comparing the components of this drug of abuse, GC-MS is a helpful technique that can help the investigator determine the plant's origin. Comparison of both extracts also aids in the understanding and acquaintance of similarities of different cannabinoids. The main objective of the study was to investigate medicinally active compounds of *C. sativa*. Results from the study proved that *Cannabis* is not meant only for its recreational purposes but the presence of these medicinal compounds in high concentrations make it a valuable source to be used in herbal medicine for different ailments.

KEYWORDS

Cannabis sativa, Cannabinoids, CBD, GC-MS

1.0 INTRODUCTION

The *Cannabis sativa* is a type of an important herbal plant, grown all over the world, belonging to the family Cannabaceae and has been used in different regions of the world as a source of oil and for medicinal purposes. According to (UNODC) United Nations Office on Drugs and Crime (2016), there are projected 182.5 million users of Cannabis globally and is extensively cultivated, marketed and used up in the form of drug. *Cannabis sativa* L. (Marihuana, Hashish) is widely grown in Pakistan as illustrated in Figure 1a, b & c. There are three species of Cannabis plant namely *C. sativa*, *C. indica* and *C. Ruderalis*, and they are found in Russia, China, India, Iran, and Pakistan. *C. sativa* and *C. Indica* are often grown all over the world while in Pakistan, they are naturally grown as the wild form in mountainous and rural areas [1].

The term ‘‘hemp’’ refers to *Cannabis sativa* cultivars grown for industrial purposes that are characterized by lower levels of tetrahydrocannabinol (THC) containing less than 0.3% tetrahydrocannabinol, the active principle responsible for Cannabis psychotropic effects. In the modern system of the breeding and cultivation of the plant for recreational and medicinal purposes, cannabis can propagate by cloning, using cuttings of a so-called ‘mother plant [2].

Cannabis sativa Linn (*C. sativa*) contains a highly complex mixture of compounds, and up to date, 568 unique compounds are identified in the Cannabis and contain about 104 Cannabinoids which are the most attractive constituents as illustrated in Figure 1 [3].

Hemp is an extraordinary crop, with enormous social and economic value, since it can be used to produce food, textiles, clothing, biodegradable plastics, paper, paint, biofuel, and animal feed, as well as lighting oil. Cannabinoids are terpene phenolic compounds produced during the growth and development of cannabis and are enriched in the hairs of the female flower glands of cannabis around the flowering stage of Cannabis. They are related to the terpenes, with their ring structure derived from geranyl pyrophosphate which represents the most specific group of compounds in this plant [4].

Phytocannabinoids accumulate in female flowers and in most aerial parts, but they have also been detected in low quantity in other parts of the plant. Generally, the concentration of these compounds depends on tissue type, age, variety, growth conditions (nutrition, humidity, and light level), harvest time and storage conditions [5].

Cannabis is the most frequently used drug of abuse not only in Pakistan but also in the whole world. Its use is increasing drastically every year. GC-MS allows for the analysis of *Cannabis sativa* which shows the differences of the constituents of this plant. Prevalence of this plant can be identified through knowledge of its constituents. In this way we can obstruct the production if we know the region in which it is produced. GC-MS is a useful technique for the comparison of constituents of this drug of abuse which will assist the investigator concerning the origin of plant. Comparison also aids in the understanding and acquaintance of similarities of different samples of cannabinoids [6].

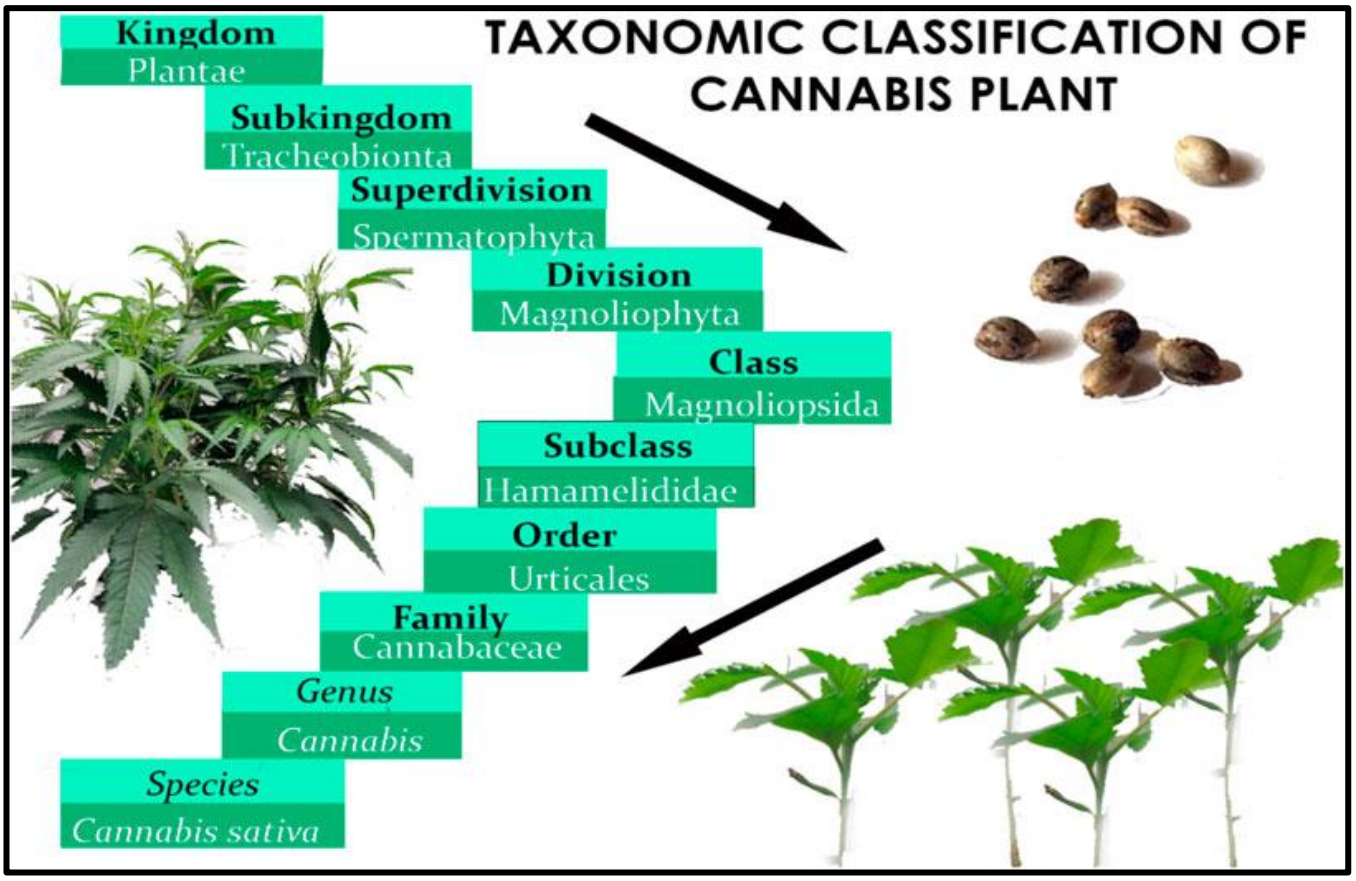


Figure 1a. Taxonomic classification of *Cannabis* plant.

Figure 1b. *Cannabis sativa* L. General aspect (a); inflorescence (b); seed (c); leaf (d); stem (e).

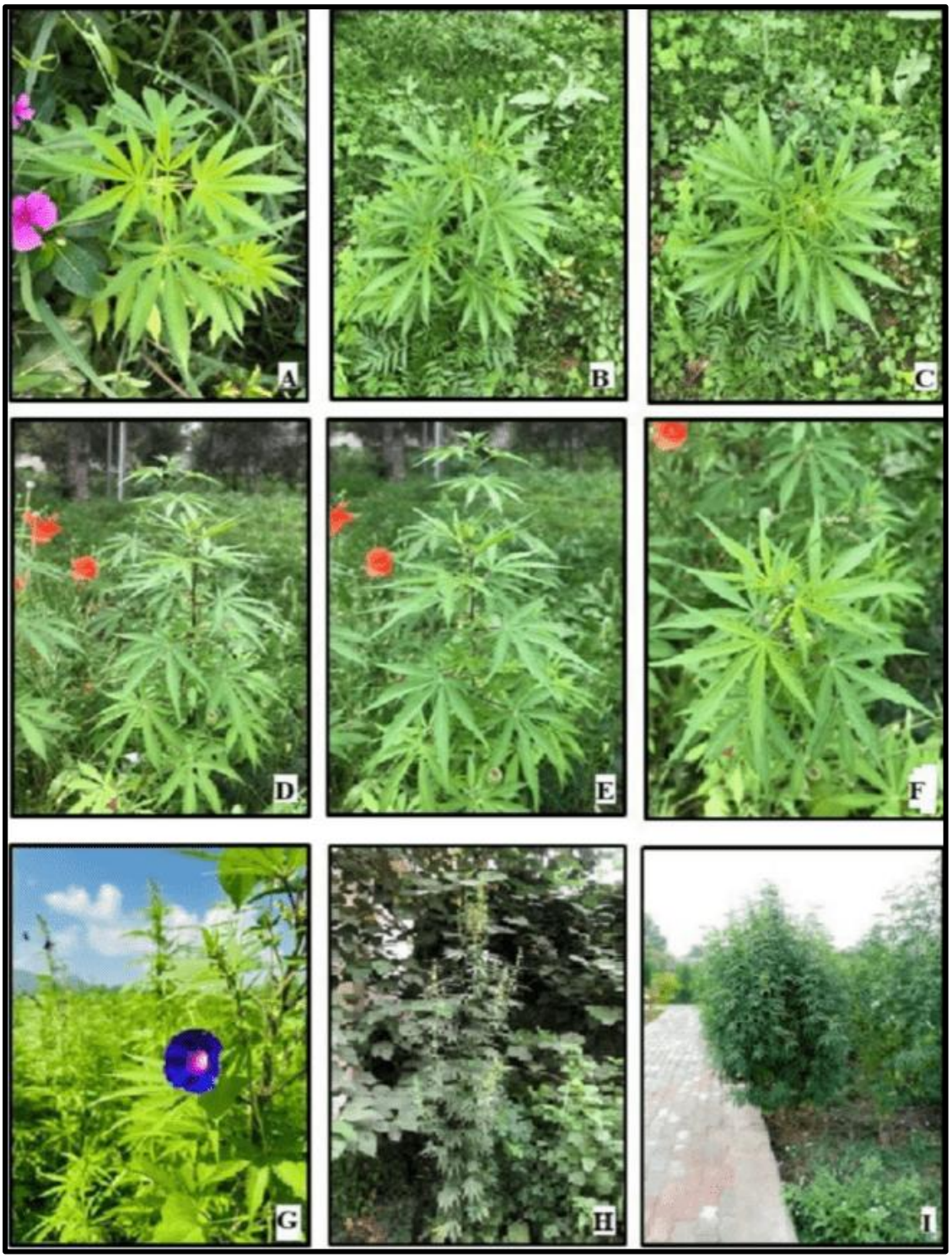


Figure 1c. Morphological representation of Immature *Cannabis sativa* (A, B, C) of Tirah Valley Variety, *Cannabis sativa* having seeds and twigs (D, E, F), Mature *Cannabis sativa* plant (G, H, I) at Medicinal Botanic Garden, PCSIR Laboratories Complex, Peshawar, KP, Pakistan.

1.2. Ethnobotanical and Medicinal Properties of *Cannabis sativa* plant:

In more than 33 different regions of Pakistan, the folk medicinal uses of *Cannabis* against ~60 ailments are still continuing [7].

Recently, an online source - the CANNUSE database was released, containing information on *Cannabis* traditional knowledge related to medicinal, alimentary, fiber and other uses from different geographical areas. This database contains 2330 data entries of *Cannabis* ethnobotanical uses from over 40 countries across the world [8].

Cannabis has been used for medicinal purposes to treat patients with various indications suitable for Cannabis therapy such as chronic pain and muscle spasticity. The drug has been proven effective in treating patients who experience severe nausea due to chemotherapy or other medicinal treatments. Studies have demonstrated the potential of cannabis as a drug that facilitates wound healing and reduces melanoma symptoms [9].

Traditionally the most common conditions for which medical cannabis is used are pain, spasticity associated with multiple sclerosis, nausea, post-traumatic stress disorder, cancer, epilepsy, cachexia, glaucoma, HIV/AIDS, and degenerative neurological conditions. Its other therapeutic uses are chronic pain, cancer, chemotherapy-induced nausea and vomiting, anorexia and weight loss associated with HIV, irritable bowel syndrome, epilepsy, spasticity, Tourette syndrome, amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, dystonia, dementia, glaucoma, traumatic brain injury, addiction, anxiety, depression, sleep disorders, Parkinson’s disease, Alzheimer’s disease, loss of appetite, post-traumatic stress disorder, and schizophrenia and other psychoses [10].

1.3. Phytochemistry (Main Cannabinoid Constituents) of *Cannabis sativa* plant:

1.3.1. Cannabidiol (CBD)

Cannabidiol (CBD) is one of the most valuable active ingredients of cannabis, and has the functions of relaxing the body and mind, protecting the nerves, and improving skin inflammation, in addition to anti-oxidation effects as shown in Figure 2a. Studies have shown that cannabidiol can be used in the treatment of many diseases, and can also effectively eliminate the hallucinogenic effect of tetrahydrocannabinol (THC) on the human body, and is known as the “anti-marijuana compound”. In many reports, cannabidiol (CBD) is a kind of cannabinoid with anti- inflammatory properties but no psychoactive effects, and has a wide range of uses in many fields. Studies relating to the use of cannabidiol as an antipsychotic drug have found that it has anti-depressant and anti-anxiety effects. For example, the FDA approved Epidiolex, a drug rich in cannabidiol, for the treatment of severe epilepsy [11].

It has been proven that it is toxic to human breast cancer cells, could slow down the metastasis and spread of cancer cells, and can inhibit neuropathic pain. Cannabidiol also has the characteristics of an anti-addiction treatment, which may be the reason it is considered to be a treatment for opioid addiction. According to many studies, cannabidiol also has a potential therapeutic effect on a wide range of neuropsychiatric diseases, and is used in the treatment of post-traumatic stress disorder (PTSD). In China, cannabidiol also shows medicinal property as an anti-cancer drug. Foods and beverages with added CBD are often used to help people manage stress, relieve anxiety, relax, lower depression and improve sleep [12].

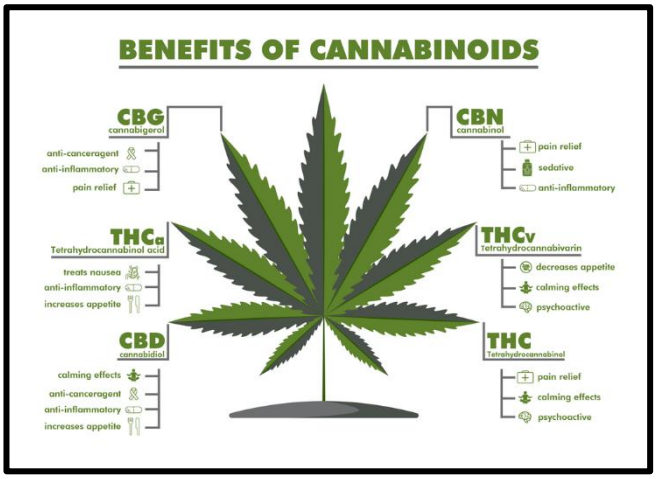


Figure 2a. Main Cannabinoids of *C. sativa*.

1.3.2. Tetrahydrocannabinol (THC)

The psychoactive component of the cannabis plant is one of the cannabinoids found in the resin glands of female cannabis stems. In order to prevent the abuse of cannabis, many countries have limited the THC content of cannabis, especially China. According to international regulations, the content of tetrahydrocannabinol in industrial cannabis is ≤0.3%. It is also an antiemetic agent which can inhibit the migration of lung cancer cells (in vitro experiments) and the growth of lung adenocarcinoma cells in vivo (oral experiments in mice) in many cases. However, on the other hand, tetrahydrocannabinol has also been proven to reduce intraocular pressure, which makes it a viable alternative to future anti-glaucoma drugs [13].

Studies have found that when tetrahydrocannabinol is used in combination with other cannabinoids, it can even relieve the neuropathic pain caused by multiple sclerosis [14].

1.3.3. Cannabichromene (CBC)

CBC is non-psychoactive and the most common cannabinoid next to THC and CBD, and is distributed in all parts of cannabis plants. Studies have shown that CBC possesses anti- inflammatory, anti-tumor, antidepressant, and antifungal properties, and also promotes brain growth. Studies have also found that CBC demonstrated anti-tumor effects against cancer cells, and a strong antibacterial effect in many cases [15].

Many studies have found CBC has analgesic, antidepressant, and anti-inflammatory effects in experimental rats [16].

1.3.4. Cannabigerol (CBG)

According to many reports, CBG can be used in the treatment of psoriasis; as an antibiotic, antidepressant, and analgesic; and has anti-tumor properties. Studies have shown that it can reduce inflammation, relieve pain, and even slow down the proliferation of some cancer cells, and that it has therapeutic potential. Studies have found that CBG is the precursor of CBGA, a precursor molecule, which in many cases can then develop into many different types of cannabinoids. According to the experience of many, CBG can prevent panic attacks faster and more effectively than cannabidiol. As reported, cannabis strains in China usually contain very little CBG, generally less than 1% by weight [17].

1.3.5. Cannabinol (CBN)

According to studies, cannabinol can be used as an antibiotic, a potential treatment for amyotrophic lateral sclerosis, and a treatment for glaucoma, and has appetite-stimulating, analgesic, anti-asthmatic, sedative, and other effects. As reported, cannabinol can usually be found in old marijuana, because tetrahydrocannabinol (THC) can be oxidized to cannabinol in the cannabis plant. Nevertheless, cannabinol is still a non-psychotropic (non-narcotic) cannabinoid. In addition, it has anti-inflammatory properties, is considered to be a medicine for treating burns, and may have an effect on bone formation. Cannabinol is usually considered to be a sedative cannabinoid, which in many cases can help people to sleep [18].

1.3.6. Cannabidivarin (CBDV)

In China, many reports have found that cannabidivarin is very similar to CBD, which is a

slightly degraded version of a cannabinoid. The research results also point out that the findings strongly support the further clinical development of CBDV in the treatment of epilepsy. Research has shown that CBDV has anti-epileptic and anti-nausea effects [19].

Some studies on autism spectrum disorder have also pointed out that the ASD (autism spectrum disorder) mouse model demonstrates the potential therapeutic mechanism of CBDV, including its potential therapeutic effects on repetitive behavior, irritability, social interaction, quality of life, and reducing inflammation [20].

However, CBDV has also been evaluated as a potential treatment for certain emotional and behavioral disorders, such as Reiter's syndrome (RETT) and autism spectrum disorder (ASD) [21].

1.3.7. Tetrahydrocannabivarin (THCV)

Research on THCV is more limited than on other cannabinoids. As with many other members of this chemical family, THCV binds to receptors located in different organs and systems, such as the brain and the immune system in many cases. In many cases, the medical value of THCV is in its anticonvulsant, weight loss, and neuroprotective effects. THCV may help regulate blood glucose levels [22].

1.3.8. Delta8-Tetrahydrocannabinol Acid (Δ8-THC)

Δ8-THC is related to Δ9-THC (dronabinol), which has very little psychoactive effect on adults, and there is currently little medical research data about either of Δ8-THC and Δ9-THC. Some sources indicate that Δ8-THC has neuroprotective and anti-anxiety properties, but more experiments are needed to verify how the cannabinoid acts in the human body [22].

1.3.9. Cannabidiolic Acid (CBDA), Tetrahydrocannabinol Acid (THCA)

According to many reports, cannabidiolic acid is the acid form found before the decarboxylation of CBD. Studies have found that CBDA has a positive effect on cancer treatment when combined with other cannabinoids, and may also have an anti-anxiety effect. A previous study revealed that they can be found in cannabis and used as a nutritional supplement or for external use. Tetrahydro cannabinolic acid (THCA) is a nontoxic carboxylic acid ("native") form of tetrahydrocannabinol found in cannabis plants. The study shows that the carboxylic acid form of tetrahydrocannabinol has unique benefits, especially in terms of anti-inflammation. The study also found that CBDA can prevent vomiting in animal models, and its binding force is 100 times that of CBD [22].

2. Experimental

2.1. Procurement of *Cannabis sativa* plant

Harvested fresh *Cannabis sativa* L. cultivated from Tirrah valley variety seeds at Medicinal Botanic Garden of Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex, Peshawar strictly for scientific research only. The botanical identities were confirmed and voucher specimens were deposited in the PES herbarium, PCSIR, Peshawar, Pakistan.

The whole plant materials of *C. sativa* (Tirah Valley) were first cleaned with running tap water and then with distilled water to remove dust and sand. These plants materials were air dried in shade for few days. The dried plant materials were crushed to fine powder in an electric blender, stored in polythene bags to avoid fungal contamination and tagged. Then different extracts were made ready by using the standard methods (Kishwar et al., 2019) [23].

2.2. Preparation of extracts using *Cannabis sativa* plant

10g plant material from each sample was grounded and soaked in respective solvents (200 ml) such as ethanol and *n*-hexane as shown in Figure 2b, filtered, concentrated under vacuum with the help of rotary evaporator maintaining the temperature between 40-50°C for 75 minutes. The extracts were then dried on water bath and stored in refrigerator.

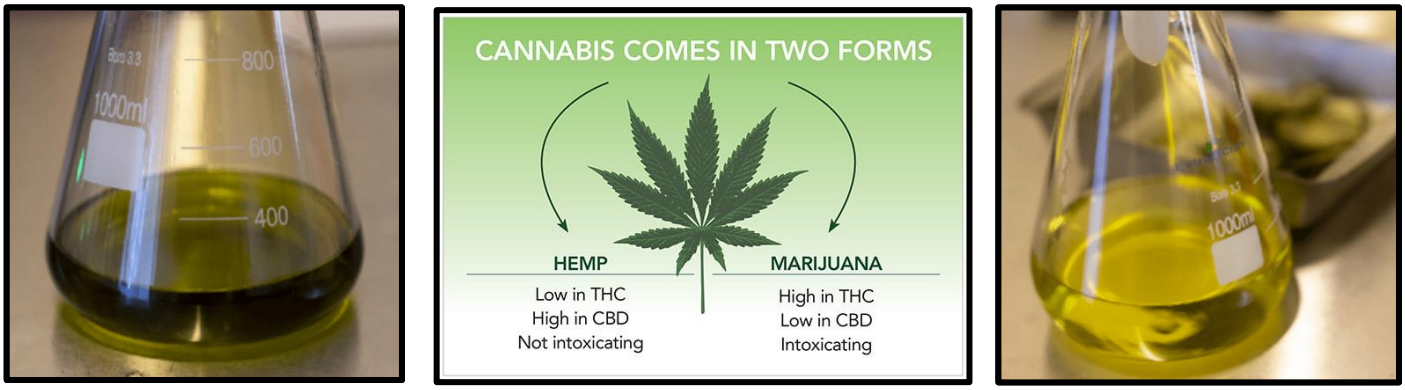


Figure 2b. Extraction of *Cannabis sativa* via Ethanol and *n*-hexane.

Extracts of the aforementioned varieties of *C. sativa* were prepared in ethanol and *n*-hexane solvent such as 1:4 (1 milligram extract: 4 mL ethanol) and *n*-hexane in 1:4 (1 milligram extract: 4ml *n*-hexane). The extracts were passed through florisil column to remove the chlorophyll. Finally, the samples were passed through filter of 0.45µm diameter and analyzed through GC-MS (Kishwar et al., 2022) [23].

2.3. GC-MS Analysis of Extract

2.3.1. Sample Preparation

From the above extract 0.2g sample was taken and stirred in 15 ml of *n*-hexane. 30ml of acetonitrile saturated with *n*-hexane was added and mixed in separating funnel. Acetonitrile layer was collected and 600ml of aqueous solution containing 2% NaCl and 100ml of *n*-hexane was added. The mixture was thoroughly mixed and organic layer was collected. Water drops present in organic layer were removed by the addition of anhydrous Na_2SO_4 and solution was filtered and evaporated to dryness. The residue was reconstituted in 1ml of cyclohexane. Solution was filtered and 1µl of the filtrate was injected into the GC-MS (Kishwar et al., 2022) [23].

2.3.2. Instrumentation (Cannabidiol, Tetrahydrocannabinol and other Cannabinoid analysis using GC-MS)

Gas chromatography coupled with mass spectrometry (GCMS) technique (Agilent USA/8890 GC System equipped with Headspace Sampler (7697A) and Automatic Liquid Sampler(7650A) and auto injector (7693A) for analysis of the aforementioned samples of *C. sativa* were carried out for quantification of CBD as shown in Figure 3. For each sample, a 2µL sample was injected in the column using automated injector in a split mode maintained at temperature of 300°C for all samples. A capillary column (DB-5ms; Technokroma, length; 30m, internal diameter; 0.25 mm, and thickness; 0.25 µm) was used with a flow rate at 1.16 mL/min for all extracts. The column oven temperature program started at 100°C for 2 minutes and changed to 300°C at the rate of 10°C/min. The column oven temperature was maintained for 10 minutes. The injection mode was normal with a washing volume of 8 µL. The flow control mode was maintained at linear velocity with a pressure of 86.2 kPa and total flow of

121.8 mL/min. MS scanning was carried out from m/z 40 to m/z 400. Ion source detector was used for the detection of analytes at temperature of 280°C. The peaks were identified by comparing the mass spectra with the National Institute of Standards and Technology library (NIST 05) (Kishwar et al., 2022) [23].

Figure 3. Analysis of CBD and other cannabinoids in *C. sativa* by GC-MS (Agilent USA/8890 GC System with 7697A Headspace Sampler and 7650A & 7693A Automatic Liquid Sampler).

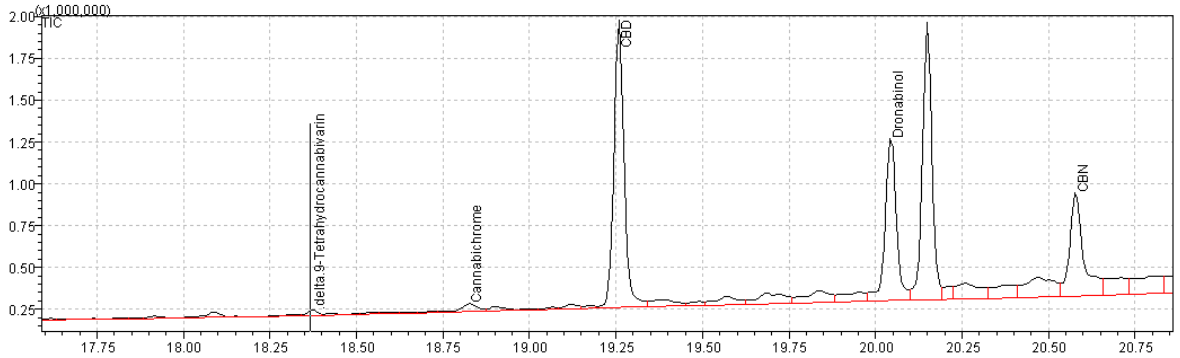
Results and Discussion

3. Results and Discussion

Gas Chromatography-Mass spectroscopy (GC-MS) is established to analyze and detect the variety of substances which are present in the sample in minute level of quantity [24-26]. The presence of cannabinoids in the supplied cannabis sample was discovered using GC-MS analysis of the samples under examination. The GC-MS analysis disclosed the presence of cannabidiol CBD and other Cannabinoids in both ethanolic and *n*-hexane extract of Tirah valley cultivar of *C. sativa*. The content of CBD detected qualitatively in *n*-hexane and ethanolic extract with percent concentrations of 80.05% and 60.58% respectively (Table 1, Fig. 4, 5). Cannabidiol is one of the major constituents of the cannabis plant. It is supposed to have a wider scope than THC because it is less psychoactive. Its molecular formula is $C_{21}H_{30}O_2$. It is used in the treatment of nausea, convulsions, bipolar disorders, etc. [27]. Interestingly, the content of THC didn't detect in both ethanolic and *n*-hexane extract respectively (Table 1, Fig. 4, 5). Tetrahydrocannabinol also known as Δ^9THC which is considered the main or principal psychoactive constituent of the cannabis plant. It is classified as schedule 1 under the convention on psychotropic substances. It is available in a synthetic form as well, its brand name is Marinol. It is a toxic component and can cause death due to over dosing [27]. The content of other Cannabinoids (CBN, Dronabinol) detected in ethanolic extract with percent concentrations of 13.62% and 6.33% respectively. The content of other Cannabinoids (CBN, Dronabinol, delta.9-tetrahydrocannabbivarin, Cannabichrome) detected in *n*-hexane extract with percent concentrations of 29.31%, 8.03%, 0.36% and 1.72% respectively (Table 1, Fig. 4, 5).

Table 1. Qualitative Percentage concentration of different cannabinoids in *Cannabis sativa* from Tirah Valley Cultivar.

S. No.	Sample	% CBD	% THC	% Other Cannabinoids			
				CBN	**Dronabinol**	**delta.9-THCV**	Cannabichrome
1.	**Ethanolic extract**	80.05	-	13.62	6.33	-	-
2.	*n*-hexane extract	**60.58**	-	**12.61**	**6.38**	**0.36**	**1.72**



ID#	Name	R. Time	Area	Conc. (%)
1	delta.9-tetrahydrocannabbivarin	18.371	5004	**0.36**
2	Cannabichrome	18.823	23968	**1.72**
3	CBD	19.257	843160	**60.58**
4	Dronabinol	20.042	111771	**8.03**
5	**CBN**	**20.577**	**407964**	**29.31**

Figure 4 (a). GC-MS analysis of cannabidiol (CBD) and other Cannabinoids in *n*-hexane extract of aerial parts of *C. sativa* Tirah valley cultivar.

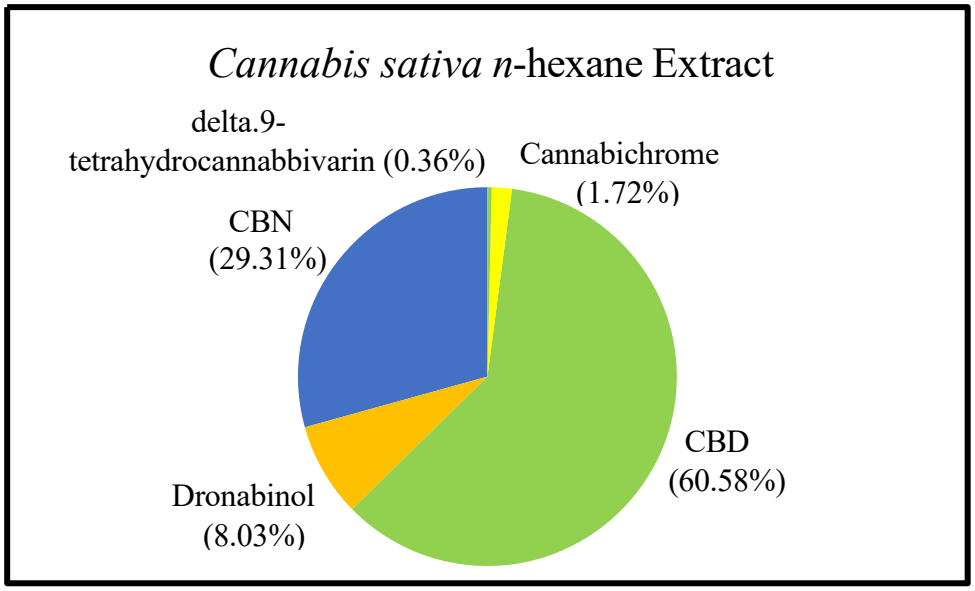
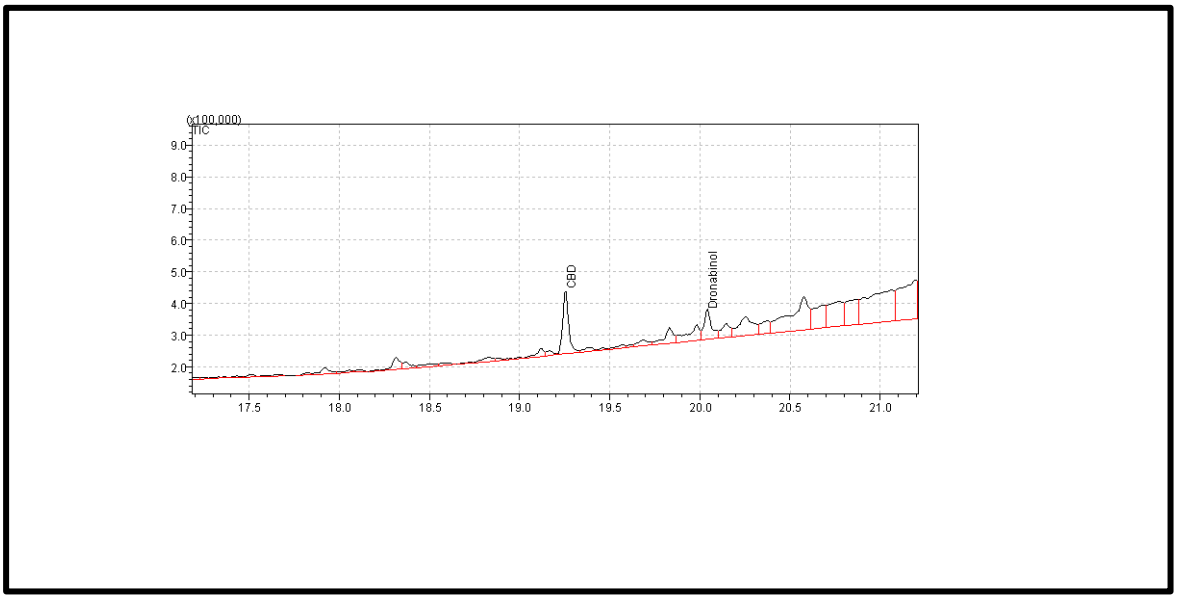


Figure 4 (b). Pie chart of GC-MS analysis of cannabidiol (CBD) and other Cannabinoids in *n*- hexane extract of aerial parts of *C. sativa* Tirah valley cultivar.



ID#	Name	R. Time	Area	Conc. (%)
1	CBD	19.253	105835	**80.05**
2	Dronabinol	20.039	8363	**6.33**
3	**CBN**	**20.639**	**18008**	**13.62**

Figure 5 (a). GC-MS analysis of cannabidiol (CBD) and other Cannabinoids in ethanolic extract of aerial parts of *C. sativa* Tirah valley cultivar.

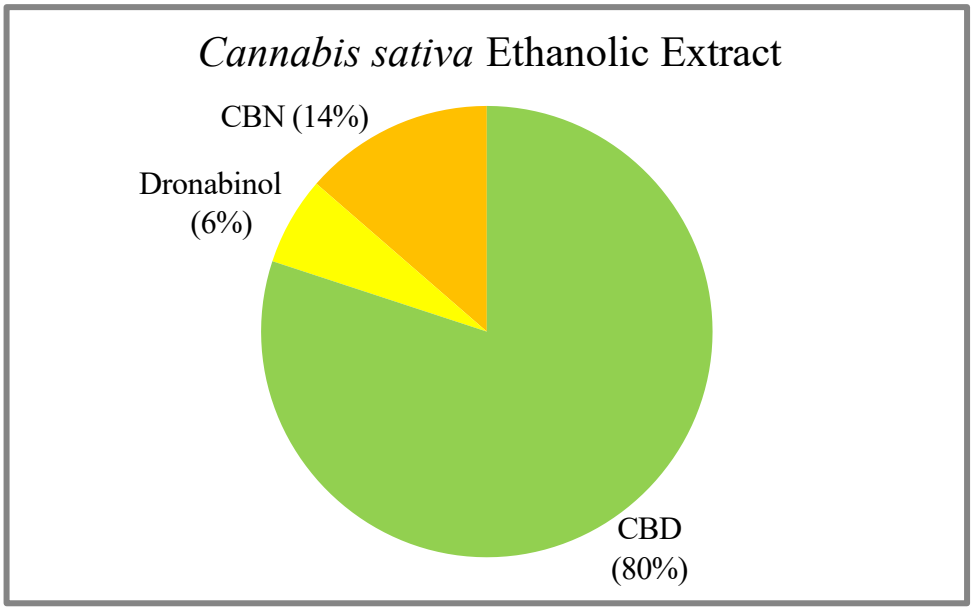


Figure 5 (b). Pie chart of GC-MS analysis of cannabidiol (CBD) and other Cannabinoids in ethanolic extract of aerial parts of *C. sativa* Tirah valley cultivar.

4. Conclusion

Cannabis contains many active compounds, of which cannabinoids are the main active components. Cannabinoids have similar molecular structures, but their real difference centers on their psychoactive properties. Unfortunately, cannabidiol, cannabinol and CBC were the only components investigated that were not psychoactive. Therefore, the use of THC, CBN, and CBC is limited to possible therapeutic purposes. There are more than 568 compounds in cannabis plants, and while individual compounds have not been studied, these are enough for us to examine the medical performance of cannabis plants. The main aim of the present study was to analyze and evaluate cannabidiol (CBD), THC and other Cannabinoids concentrations in the ethanolic and *n*-hexane extracts of Tirah Valley cultivar of indigenously grown *Cannabis sativa* with the help of GC-MS analysis in which maximum CBD is detected in both extracts along with other cannabinoids.

However, most of the industrial cannabis planted in other developed countries is used for fiber, with little or no CBD. Cannabidiol is the main non-psychoactive component of cannabis, and its anti-tumor activity *in-vitro* and *in-vivo* is considered to make it an anti-tumor drug. As the applications of cannabidiol in the fields of medicine and healthcare are gradually being recognized by the market, industrial cannabis and related industries have broad development space. Therefore, commercial scale productivity, yield and quality enhancement of the CBD is highly encouraged and appreciated to boost the country economy and increase its worth in the international market.

In the early twentieth century, cannabis was overlooked due to availability of alternate drugs, lack of quality control, risk of abuse and intoxication. The change in public and political opinion has directed the leadership to implement medical cannabis legalization. Although it is legally approved in several regions, still safety data from clinical trials of cannabis is not as much robust as for other pharmaceuticals. Currently, several countries including United States, Canada, Taiwan, Pakistan and many others are focused on the medicinal values and uses of cannabis. In near future, cannabis would be considered an attractive source of revenue for those who acquire quality medicinal products from it and commercialize them in national and international markets.

Conflict of Interest

The authors have no conflicts of interest regarding this investigation.

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