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Microscopical and Chemical Study of *Cannabis sativa*

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Abstract

Now a day's cannabis is enormously using as for the drug abuse in India including other developing countries. The plant *Cannabis sativa* is also known as marijuana, presents unique issues in our justice system, not the least of which is its identification both by law enforcement officers as well as Forensic Crime Laboratories. Present work is aimed for identification of that biological evidence of Cannabis plant, which can enhance the suitability of identification. Comprehensive studies of both chemical and morphological methods have led investigators to conclude that the two approaches are complementary [1]. In this morphological study was performed by using trinoccular biological research microscope while chemical study by colour/spot tests and Thin Layer Chromatography due to its ease of availability and handling. A combination of the two provides experienced analysts with a very reliable means for identifying Cannabis fragments. If any of the residue/fragments of Cannabis plant found at the crime scene, a Forensic Analyst (investigator) can adopt any of the identification technique as suitable as for their reliability [2]. Some of the researchers have been already qualified these types of techniques but present study included the anatomy of root, stem, with their visual feature followed by chemical and instrumental techniques which can provide accuracy of identification.

Keywords: *Cannabis sativa*; Anatomy; Tetrahydrocannabinol; Cannabinol; Duquenois-Levine; Fast blue-B

Introduction

Cannabis is a tall upright annual herb. It is generally dioecious i.e. producing separate male and female plants but fiber hemp varieties have been specifically bred to be monoecious (hermaphrodite) [1,2]. The leaves are palmate, and in the iconic image of a cannabis leaf there are seven lobes, the lowest pair showing as backwards facing spurs. However, this number and shape is not fixed. On seedlings the first pair of leaves is typically monophylous (single lobed), the second pair having three lobes and the next pair five. In many plants, especially of central Asian origin, the number does not extend beyond five while in others the number can extend to around thirteen [3].

Figure 1: Clustere of Seeds of Cannabis Sativa.

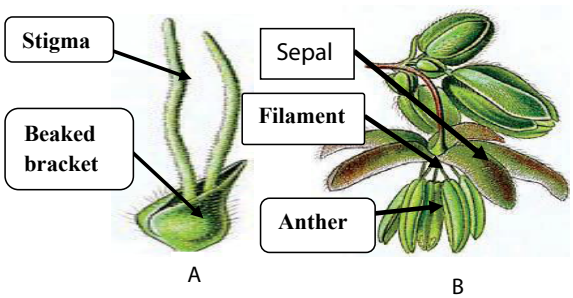

Figure 2: Morphological characteristics of (A) female and (B) male flower's.

The genera Cannabis and Humulus (hops) belong to the same family (Cannabaceae, sometimes known as Cannabinaceae). Generally, cannabis is considered to be monospecific (*Cannabis sativa* L.) which is divided into several subspecies (*C. sativa* subsp. sativa, *C. sativa* subsp. indica, *C. sativa* subsp. ruderalis, *C. sativa* subsp. spontanea, *C. sativa* subsp. afiristanca). However, the chemical and morphological distinctions by which cannabis has been split into these subspecies are often not readily discernible, appear to be environmentally modifiable, and vary in a continuous fashion. For most purposes, it will suffice to apply the name *Cannabis sativa* to all cannabis plants encountered [4] (Figures 1 and 2).

Cannabis is an annual, dioeciously, flowering herb. Staminate (male) plants are usually taller but less robust than pistillate (female) plants. Stems are erect and can vary from 0.2-2.0 m. However, most of the plants reach heights of 1-3 m. The extent of branching, like the plant height, depends on environmental and hereditary factors as well as the method of cultivation. Cannabis is a tall, erect, annual herb, provided with an open sunny environment, light well-drained composted soil, and ample irrigation [5]. Cannabis will deteriorate in about two years if exposed to light, air or heat. It should always be stored in cool places (Figures 3 and 4).

Cannabis is a wind pollinated species. The males, which are generally taller than the females commence flowering first. The plant was grown in still conditions and leaves appear yellow under the deep

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Figure 3: Abaxial (left) and adaxial (right) of Cannabis-Sativa leaves surfaces.

Figure 4: A part of Stem of Cannabis Sativa.

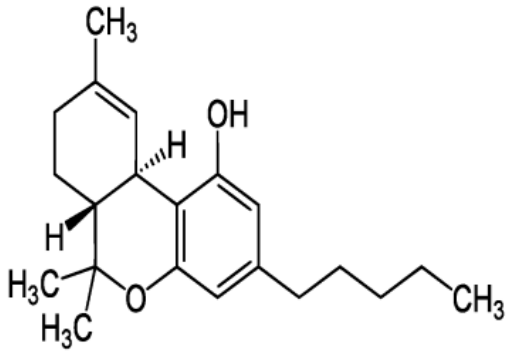


Figure 5: Tetrahydrocannabinol(THC).

covering of pollen. When mature, the sepals on the male flowers open to expose the anthers, which hang freely on fine filaments. The female plants tend to be shorter and have more branches than the male. Female plants are leafy to the top with many leaves surrounding the flowers, while male plants have fewer leaves near the top with few if any leaves along the extended flowering limbs.

Products of cannabis

The sticky resin produced by the flowers and top leaves contains a number of psychoactive substances, collectively known as cannabinoids, these collectively make up the drug called cannabis. The potency of the cannabis obtained from a plant is dependent on the content of delta-9-tetrahydrocannabinol (THC), the most important of the cannabinoids [6]. THC content is dependent on the part of the plant used, the method of cultivation, and the preparation of the extract:

- **Bhang** obtained from cut tops of uncultivated plants with low resin content is the least potent.
- **Ghanja** or marijuana from flowering tops and leaves from specially cultivated plants has higher resin content and is more potent. Both of these herbal preparations (also known as 'grass' or 'weed') are usually smoked in hand-rolled cigarettes ('joints' or 'reefers'). Potency is variable, with a THC content of 1-10 per cent.
- **Cannabis resin (hashish)** is the resin itself, in the form of a sticky brown cake, which can be smoked or eaten.
- **Liquid cannabis or hashish oil** is extracted from cannabis resin, and is more potent. Tobacco is dipped in this before smoking. It may contain up to 60 percent THC, and is a Class A drug.

The Cannabis plant and its products consist of an enormous variety of chemicals. Some of the 483 compounds identified are unique to Cannabis, for example, the more than 60 cannabinoids, whereas the terpenes, with about 140 members forming the most abundant class, are widespread in the plant kingdom. Cannabis contains over 300 compounds. At least 66 of these are cannabinoids, five important cannabinoids found in the cannabis plant are:

1. Tetrahydrocannabinol (THC)
2. Cannabidiol (CBD)
3. Cannabinol (CBN)
4. β-caryophyllene
5. Cannabigerol.

Tetrahydrocannabinol (THC): Tetrahydrocannabinol (THC) is the primary compound responsible for the psychoactive effects of cannabis. The compound is a mild analgesic, and cellular research has shown the compound has antioxidant activity.THC is believed to interact with parts of the brain normally controlled by the endogenous cannabinoid neurotransmitter, anandamide. Anandamide is believed to play a role in pain sensation, memory, and sleep (Figure 5).

Cannabidiol (CBD): Cannabidiol (CBD) is a major constituent of medical cannabis. CBD represents up to 40% of extracts of the medical cannabis plant. Cannabidiol has been shown to relieve convulsion, inflammation, anxiety, cough and congestion, nausea, and inhibits cancer cell growth. Recent studies have shown cannabidiol to be as effective as atypical antipsychotics in treating schizophrenia. Because cannabidiol relieves the aforementioned symptoms, cannabis strains with a high amount of CBD may benefit people with multiple sclerosis, frequent anxiety attacks and Tourette syndrome (Figure 6).

Cannabinol (CBN): Cannabinol (CBN) is a therapeutic cannabinoid found in *Cannabis sativa* and *Cannabis indica*. It is also produced as a metabolite, or a breakdown product, of tetrahydrocannabinol (THC). CBN acts as a weak agonist of the CB1 and CB2 receptors, with lower affinity in comparison to THC (Figure 7).

β-Caryophyllene: Part of the mechanism by which medical cannabis has been shown to reduce tissue inflammation is via the compound β-caryophyllene. A cannabinoid receptor called CB2 plays a vital part in reducing inflammation in humans and other animals. β-Caryophyllene has been shown to be a selective activator of the CB2 receptor. β-Caryophyllene is especially concentrated in cannabis essential oil, which contains about 12-35% β-caryophyllene (Figure 8).

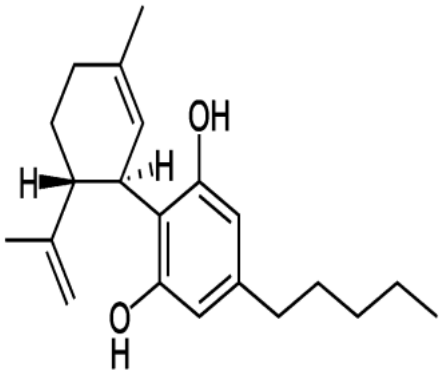


Figure 6: Cannabidiol (CBD).

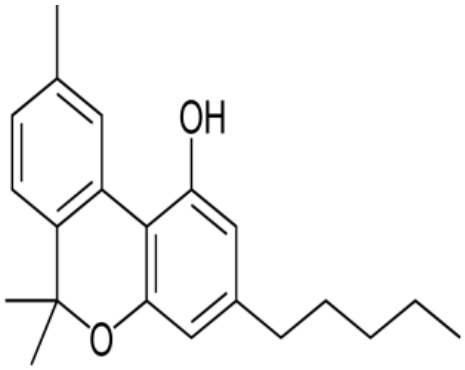


Figure 7: Cannabinol (CBN).

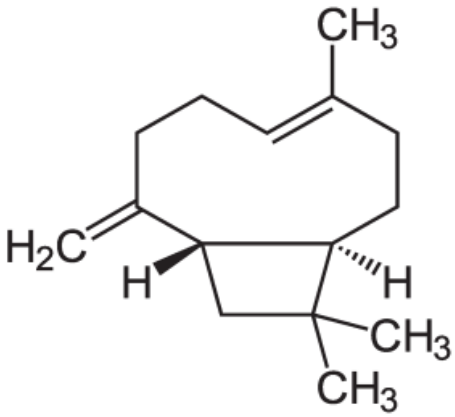


Figure 8: β- Carophyllene.

Cannabigerol: Like cannabidiol, cannabigerol is not psychoactive. Cannabigerol has been shown to relieve intraoccular pressure, which may be of benefit in the treatment of glaucoma (Figure 9).

Material and Methodology

Cannabis plant is found in most of the countries and cultivated also for the medical purposes. But in the present study *Cannabis sativa* of Bundelkhand region of Uttar Pradesh was collected for examination. Bundelkhand is spread over southern Uttar Pradesh (UP) and northern Madhya Pradesh (MP), between 23°10' and 26°30' north latitude and 78°20' and 81°40' east longitude. The region covers a geographical area of around 70,000 sq km and includes seven districts of UP and six districts of MP. The northern part of Bundelkhand, almost entirely in UP, is a flat plain. In the central and southern part are rocky outcrops, stepped Vindhyan plateaus that rise 300 to 450 m above sea level, and broken hill ranges up to a height of 600 m. Hill ranges are prominent in Panna and Damoh districts, in the southeast, and Sagar district, in the southwest. All major rivers of the region flow from south to north, emptying into the Yamuna.

Samples are collected as a whole plant with their roots, very soft tips, leaves (mature and newly evolved), flowers, seeds, and stem. Stem collected for anatomical study that is why soft stem were collected. Root collected from soft plant and washed. The width of stem was not more than 5 mm and for root it was not more than six mm. Root was cleaned thoroughly to remove soil and other unwanted particles attached with them. Leaves and other parts of plant were also cleaned by distilled water and unuseful material was discarded. Leaves and plant tips along with flower were dried for about 10-15 days prior to use for study while materials for microscopic examination preserved in the formalin [7].

Microscopic examination of *Cannabis sativa*

Microscopic examinations have great significance in the identification of *Cannabis sativa*. In the present study root, stem and leaves are studied by the microscope. In the present study trinoccular Biological Research Microscope (RS41 OLYMPUS) was used. Its greater advantage in use was an attached digital camera to capture image developed by microscope. A light source enhances the contrast and gives clear and easily visible image of the specimen. Intensity of light can be increased or decreased by a knob situated at the below side on microscope. Specimen can be focused best in the line of light rays by rotating a knob, which rotates the platform right-left and front-back. There is no need to touch again the slide for adjustment.

It can magnify image upto 5×, 10×, 45× and 100×. Resolution can be set as better as we need at each magnification. Three ocular lenses present in a broad tube upon which camera is fixed to take digital photograph. This microscope is much better than the simple compound microscope to study the anatomical view of transverse or longitudinal sections (Figure 10).

Anatomical examination of root: In present study anatomical examination was carried out by transverse sections of root of *Cannabis sativa*. Fresh plant's root was used for sectioning. A sharp blade was used to cut the transverse sections of root. Several sections cut for the staining and a permanent double stained slide prepared by passing the alcohol series (i.e. 30%, 50%, 70%, 90%, 100% pure ethanol). Firstly washed the specimen properly and stained with Safranin followed by Light

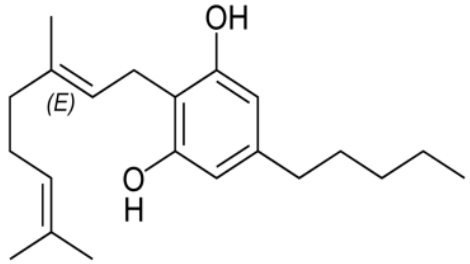


Figure 9: Cannabigerol.

Figure 10: Biological research trinoccular microscope.

green combination and passed from alcohol series. Finally mounted the sections, fixed cover slip by DPX (Digital Picture Exchanger) mountant and left for dryness. On observing under Biological Research Microscope, taken several pictures of stained sections for study. The pictures are taken using 5× and 10× eye piece for better identification.

Anatomical examination of stem: Texture, shape and morphology are also considered for examination. But a transverse section was studied for more valuable consequences not more than 5 mm in diameter. Washed thoroughly by distilled water and cut several fine vertical sections for mounting and staining. Placed the sections into watch glass and washed it by distilled water. Stained first with Safranin and washed again to remove excess color and then with Light green. Alcohol series was also applied to mount that slide. Distilled water used again to moisture the cells and taken on glass slide followed by DPX mountant for fixing the cover slip. Observed slide under microscope explained above and taken several pictures for study.

Anatomical examination of leaf: Leaves have individual characteristic on its surface, margin and veins. Many fine hairs were present on the upper and lower surface. The leaves residue or a fine piece of leaf was taken on microscopic slide and 2 drops of 0.1 N NaOH solution was poured on material and left for 10-12 minutes. After fixing cover slip upon the material, observed by microscope with 5×, and 10× eye piece.

Chemical examination of *Cannabis sativa*

Several chemicals or color tests were performed to give better observation report. The color tests adopted were Duquenois-Levine test and Fast Blue- B salt test [8].

In Duquenois-Levine test, Duquenois reagent was freshly prepared by adding 0.4 gm vanillin and 0.5 ml acetaldehyde in 20 ml ethanol (2.5% v/v of acetaldehyde and 2%vanilline w/v in 95% ethanol). Placed a small amount of the suspect material in a test tube and shaked with 2 ml of Duquenois reagent for 1 minute. Added 2 ml of concentrated hydrochloric acid and shaked the mixture. Allowed to stand about 10 minutes followed by addition of 2 ml of chloroform and mixed gently [9].

In Fast Blue-B salt test, pulverized sample of cannabis (i.e. leaves and flower tips) was taken into the test tube and 3 ml of Fast Blue-B salt (Fast blue-B salt mixed with anhydrous sodium sulphate in the ratio of 2.5:1) was added followed by 2 ml of chloroform. Later 0.1 N aqueous NaOH was added and left for 2 minutes. A wine red color was developed.

Thin layer chromatographic analysis: In present study, glass plate (10 × 4 cm) and silica gel G (Merck Chemicals Pvt. Ltd. Mumbai), was used to prepare TLC plates for the identification of *Cannabis sativa*. Several steps were followed as TLC plate formation, chamber preparation, plate activation etc. required for chromatographic process. Silica gel coating were prepared in distilled water with the ratio of ratio of 1:2 of powder and water. Dilution of silica gel in water should be very careful as it relates the thickness of coating. The preparation of gel requires constant stirring. Pouring method was adopted for coating plate [10].

After pouring the gel, plate was placed into an oven to dry completely at 110°C for 1 hour. After complete dryness, the plate was left for overnight in environmental temperature to activate. Then the plate was ready to use for spotting.

Extraction of sample: Several methods for extraction of sample were available but in present study Cannabis material was prepared by dissolving the plant material in Carbon Tetrachloride (CTC) as 0.1 mg/ml. Prepared material was kept for vaporizations of CTC. But gently heated the solution to evaporate and again dissolved the dried residue in 2 ml of methanol. Thoroughly mixed the solution and used for spotting.

Plates were loaded by sample with the help of capillary tube. The plates were again dried and placed into chamber. The chamber was pre-saturated with solvent system (S_1) Chloroform and Methanol (50:50). One more solvent system (S_2) Ethyl alcohol:Methanol:Ammonia (8.5:1.0:0.5) also used to find out the variation of Rf values in different mobile phases. However, the sample extraction procedure for these solvent was different and extracted in chloroform (1 mg/ml, w/v). Cannabis plant material were soaked in Chloroform and evaporated to dryness and residue was reconstituted in 1 ml methanol.

TLC plates were developed in chamber and when the solvent reached upto 2/3 of plate, these were removed from chamber and dried. Then the plates were observed under U-V light (Wavelength 254-360 nm). Sprayed fast Blue-B salt reagent to develop the color spots on plate.

Preparation of fast blue-B salt solution: 50 mg of fast blue-B salt powder (Merck Chemicals Pvt. Ltd.) was dissolved in 1 ml distilled water. Mixed thoroughly, added 20 ml of methanol in it, and stirred properly. A light brownish color solution was developed.

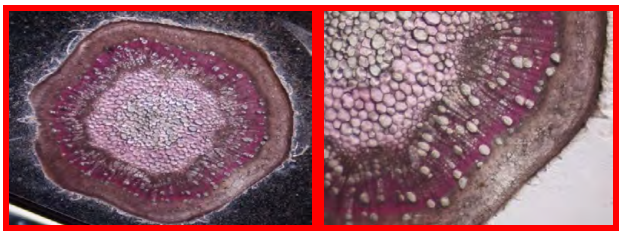
Taken several photographs of plate often visualization the color spots on plate and measured the distance of solute and solvent from its beginning point where sample was loaded. The Rf value was calculated. Different spots were observed and compared. The Rf value calculated showed the presence of constituents of cannabis plant.

Results

Cannabis sativa was identified by using different microscopic, chemical and instrumental techniques. Microscopical study of Transverse section of Root and Stem showed the characteristics of dicotyladons [11].

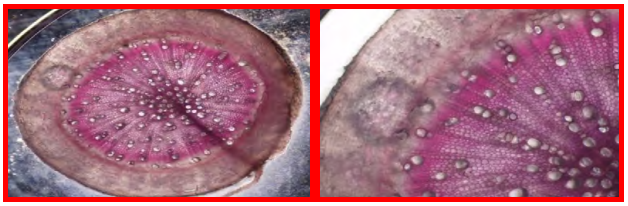
Cuticle observed and hairs were present on the cuticle. Regular and radial pattern of cortex and xylem, phloem were observed. Pericycle was also observed beneath the cortex and Pith was seen in the central region of Transverse section of stem (Figure 11) [12].

T.S. of root (Figure 12) also showed the regular pattern of cortex below epidermis. Metaxylem was also observed. Green and red stain separated the arrangement of cells.



(A)- Whole section (B)- A part of section

Figure 11: (A & B)-Transverse section of Stem of Cannabis Sativa.



(A)- whole sect (B)- a part of section

Figure 12: (A & B) - Transverse Section of Root of Cannabis Sativa.

Chemical color tests were also given the positive test. When the sample treated with Duquenois-Levine reagent followed by HCl and Chloroform, purple color appeared in the chloroform layer. While if treated with fast blue-B salt in addition of other reagents, wine red color appeared in the chloroform layer. Results of chromatographic examination was observed from each plate and calculated the Rf values with different solvent system. Different colored spots were developed on plate was in different manner when shifted in different solvent systems. As in chloroform and benzene solvent system, pink, violet, brick-red color was developed that confirms the presence of Cannabinol, Cannabidol and Tetrahydrocannabinol respectively. Likewise in ethyl acetate: methanol: ammonia; red, orange and purple spots were observed that shows the presence of Tetrahydrocannabinoic acid (THC-COOH), Cannabidol (CBD) and Cannabinol (CBN). Rf values calculated after visualization of spots on the plates are given below.

Table 1 describes the Rf values for consecutive samples i.e. A, B and C. There 3 spots were found on each plate with samples A, B and C, when developed in Chloroform and Benzene in the ratio of 50:50 respectively. Rf values were found as 0.195, 0.435 and 0.798 with Pink, Violet and Grayish-violet colour spots in respect to Rf values. Likewise Rf values 0.192 (Pink), 0.440 (Dark-red) and 0.804 (Orange-pink) were observed from sample B and 0.189 (Pink), 0.448 (Dark-violet) and 0.795 (Orange-pink) from sample C.

Table 2 describes the range of variation of Rf values for each compound as Cannabinol (CBN), Cannabidiol (CBD) and Tetrahydrocannabinol (THC) in Chloroform and Benzene. Mean value was calculated from three Rf values of each samples of Cannabinol i.e. 0.192 and range of variation was -0.003 to 0.003. Likewise mean of Rf value was 0.441 for Cannabidiol while range of variation was -0.006 to 0.007 while for Tetrahydrocannabinol, the mean of Rf values was 0.799 with -0.004 to 0.005 range of variation.

Table 3 describes the Rf values for three sample i.e. A, B and C. There were three spots were found on each plate with samples A, B and C. When developed in ethyl acetate, methanol and Ammonia in the ratio of 8.5:1.0:0.5 respectively. Rf values were found as 0.308, 0.955 and 0.984 with Red, Orange and Purple colour spots with respective Rf values. Likewise Rf values 0.310 (Pink), 0.948 (Orange) and 0.980 (Purple) were observed from sample B and 0.318 (Red), 0.951 (Orange), and 0.795 (Purple) from sample C.

Table 4 describing the range of variation of Rf values for each compound as Cannabinol, Cannabidiol and Tetrahydrocannabinol in Ethyl acetate, Methanol and Ammonia. As mean taken from three Rf values of each sample for Cannabinol was found 0.313 and range of variation was -0.004 to 0.006. Likewise mean of Rf values for Cannabidiol was 0.952 with -0.003 to 0.004 range of variation while 0.982 mean and -0.002 to 0.002 range of variation were found for Tetrahydrocannabinol.

As values are indicating the similar Rf value in slight variation this may be due to some manually experimental errors. Rf values are calculated upto three decimal to validate the results. Three samples were performed in each solvent system and three spots were observed on each plate but color differentiation was very difficult due to merging of colors. It was possible for only short time after spraying and colors were going to fade as passes the time. A line of colors were visible after some time and below pictures are only samples of visualized plate.

Sample No.	Spot No.	Rf Value	Color of spots
A	1	0.195	Pink
	2	0.435	Violet
	3	0.798	Grayish-violet
B	1	0.192	Pink
	2	0.440	Dark-violet
	3	0.804	Orange-pink
C	1	0.189	Pink
	2	0.448	Dark-violet
	3	0.795	Orange-pink

Table 1: Table showing Rf values in Chloroform:Benzene with color spots.

S.No	Constituents	Rf values			Mean of Rf values	Range of variation of Rf values
		1st	2nd	3rd		
1	Cannabinol	0.195	0.192	0.189	0.192	-0.003 to 0.003
2	Cannabidiol	0.435	0.440	0.448	0.441	-0.006 to 0.007
3	Tetrahydrocannabinol	0.798	0.804	0.795	0.799	-0.004 to 0.005

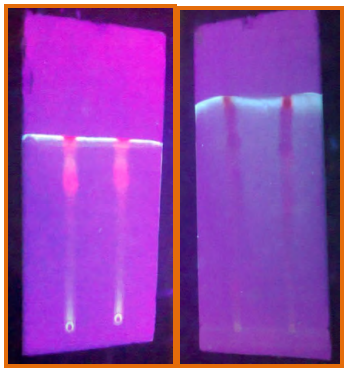
Table 2: Range of variation of Rf values for different compound in Chloroform and Benzene.

Sample No.	Spot No.	Rf Value	Color of spot
A	1	0.308	Red
	2	0.955	Orange
	3	0.984	Purple
B	1	0.310	Red
	2	0.948	Orange
C	1	0.318	Red
	2	0.951	Orange
	3	0.980	purple

Table 3: Table showing Rf values in ethyl acetate, methanol and ammonia.

S. No	Constituents	Rf values			Mean of Rf values	Range of variation of Rf values
		1st	2nd	3rd		
1	Cannabinol	0.308	0.310	0.318	0.313	-0.004 to 0.006
2	Cannabidiol	0.955	0.48	0.951	0.952	-0.003 to 0.004
3	Tetrahydrocannabinol	0.984	0.981	0.980	0.982	-0.002 to 0.002

Table 4: Range of variation of Rf values for different compound in ethyl acetate, methanol and ammonia.



(A) (B)

Figure 13: TLC plate developed in (A) chloroform and benzene and (B) Ethyl acetae, methanol and ammonia.

Spots on above plates (Figure 13) are explaining the separated colours of different compound which are mainly present in *Cannabis sativa*. Colours are not clearly visible in above figure due to some fadedness after passing the time but these can be determined at some extent.

RAPD markers analysis

RAPD markers were used to individualize Palo Verde tree in a criminal case and strawberry in a civil case [13,14]. In both cases the method has been accepted in court although, in the Palo Verde tree case the statistical significance was not used since the representative population consists of too few samples. Congiu et al. [14] employed RAPD markers for individualization of strawberry because of its two main advantages: it allows random sampling of markers over whole genomic DNA and does not require any previous information on the genome of the organism under estegation inv. Although RAPD marker analysis has reproducibility problem, it is inexpensive, simple to perform, and has moderate ability to distinguish between unrelated individuals compared to AFLPs and STRs [15]. Therefore, the method can still be useful for individualization of Cannabis samples in the developing countries that have very limited lab facilities but majority of Cannabis production occur.

So we can say that RAPD marker analysis is important for Cannabis: (1) to analyze the high number of seized Cannabis samples by means of RAPD, (2) to compare two different approaches (in the first, a single plant represents an accession and in the second, a set of ten different plants of the same accession bulked equally represent the specific accession) for individualizing Cannabis accessions, (3) to obtain information on the genetic variation and relatedness which might be a useful information about the sources and distribution networks of these illicit substances.

Conclusion

As cultivation of *Cannabis sativa* plant is banned in India as well as in some other countries however it can be cultivated after achieving a license by government. Present work is useful for systematic and regular identification of cannabis plant and to differentiate from others. It is not necessary to find out whole plant at the crime scene but its residue like stem, root, leaves flowers etc. may be found either separately or together. If only stem or root was found, anatomical examination can be performed. If leaves were found, microscopical and chemical tests including TLC can be adopted to identity the leaves, because presence of constituents of plants in leaves confirm the identity. If flower plant tips and seeds are found, these can be identified by using physical observation, chemical tests and thin layer chromatography. If whole plant is found, systematic examination can be followed which are mentioned in present work. Furthermore statistical calculation of Rf values concluded that the chances of error in chloroform and benzene for Cannabinol (CBN) is lesser while more in ethyl acetate; methanol and ammonia. Likewise, Cannabidiol (CBD) and Tetrahydrocannabinol (THC) show lesser chance of error in ethyl acetate: methanol and ammonia while more in chloroform and benzene solvent systems.

This paper is most helpful and advantageous as it includes several identification techniques of *Cannabis sativa*. One can use any of the method or all described in present paper. Several other methods as GC, GC-MS, HPLC, Spectrophotometers can also be adopted for expanded result. Some other quantitative and qualitative methods can also be applied after explained methods.

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