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Research Article

Botanical, Chemical and Microscopical Comparative Study of Two Chemotypes of *Cannabis sativa* Growing in Morocco (Province of Taounate)

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ABSTRACT
The cultivation of cannabis in Morocco is an ancient traditional culture, mainly in the North region. The present work aims at undertaking a comparative study on botanical, microscopic and chemical aspects of two chemotypes of *cannabis sativa* (Kif and Khardala) growing in three sites in Taounate region in the north of Morocco (Khlalfa, Tafrant and Oudka). The morphological study was performed by using binocular magnifying glass and biological research microscope and a chemical study by Thin Layer Chromatography (TLC) due to its good separation and ease of availability and handling. Indeed, TLC method has been applied in this work for detecting and isolating a various components of plant of Cannabis female inflorescences. Regarding the botanical and microscopic examinations, there is not a significant difference between the two chemotypes. However, chemical analysis by TLC revealed some differences in quantity and quality of Cannabinoids compounds. These data suggest a botanical, chemical and microscopic comparative study of two chemotypes of Cannabis *sativa* growing in Morocco in order to provide an accurate identification.

Keywords: morphology, microscopic study, TLC, Kif, Khardala, *Cannabis sativa.*

INTRODUCTION

Cannabis, the plant that produces hemp as well as hashish, is now known primarily as one of the leading psychoactive plants in world use, following tobacco and alcohol. Probably one of the oldest plants used by human being, cannabis was cultivated for fiber, food and medicine thousands of years before it became the "superstar" of the drug culture[1]. In Morocco and particularly in Taounate region, despite efforts provided by the local authorities, often by force, to abolish cannabis, this traditional culture has resisted all approaches and has spread to other parts of the country. The precarious socio-economic situation of farmers and the lack of adequate agricultural policy are the most significant causes of the spread of this plant. Furthermore, three chemotypes of *cannabis sativa* are growing in Taounate region (Kif, Khardala and Pakistana). However, this study has focused only on two chemotypes, kif "a local variety" (not irrigated) and Khardala "introduced variety" (strongly irrigated). *Cannabis sativa L.* has a long history as a recreational drug but it has also been used as a traditional medicine in many cultures. Despite the therapeutic potential of cannabis its classification as a narcotic prevented its development in modern medicine. The psychoactive cannabinoid, known as tetrahydrocannabinol (THC) has received much scientific attention. Several studies demonstrated the potential of C*annabis sativa* in treatment of various dysfunctions and diseases. Indeed, *Cannabis Sativa* has an anti-edematous effect, antidepressant and a great effectiveness in reducing anxiety in patients suffering from social anxiety disorder[2-4]. The immunomodulatory effect is also present in the illicit plant through the use of unheated extracts which contain mainly THCA[5]. Colon cancer fighters were found also in Cannabis extract with high quantity of CBC and BDS (cannabidiol botanical drug substance) by inhibiting cancer cell proliferation via activation of CB1 and CB2[6]. Our present study aims at highlighting the difference between Kif and Khardala based on the botanical, microscopic examination of cannabis powder and chemical study by using TLC method.

Overview

Cannabis sativa L. is a dicotyledonous herb to taproots (Fig.1), apetal, dioecious, the order of Urticales, family Cannabinacea. This is an annual herb that can reach 2m for Khardala and 1.5m for Kif. Males have slender legs; they are easy to differentiate from female feet (Fig. 2 and 3).

Cannabis Culture and Harvesting

**Author for Correspondence*

Figure1. Root of Cannabis *Sativa L*

Figure 2. Inflorescence of male Cannabis *sativa L.*

Figure 3. Inflorescence of a female Cannabis *Sativa L.*

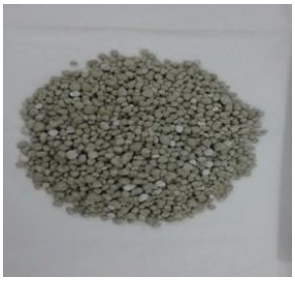


Figure 4. Complex granulated fertilizer

Figure 5. Optical microscope with external light

The culture of Kif is in March and its harvesting at the end of June or early July. Its growth does not require irrigation. However, Khardala and Pakistana are cultivated in April and its harvesting is in October. They require a lot of irrigation to have a good yield. The farmers prefer to add mineral fertilizers that refer to the complex granulated fertilizer containing 18% nitrogen (N) in the ammonium form and 46% of phosphoric anhydride (P_2O_5) soluble in water and ammonium citrate (Fig.4). It is interesting to note that irrigation must be stopped 15 days before harvesting. It allows a good yield of hashish resin (personal communication).

For harvesting, usually when a flower matures, it withers and turns brown. When about 75% of stigma became brown, the plants are ready to be harvested[7]. The following table summarizes the points of difference between these 2 chemotypes of cannabis.

Table 1. Main differences between Kif and Khardala

	Kif	Khardala
Culture	March	April
Harvest	In late June or early July	October
Irrigation	Unirrigated	Irrigated
Fertilizer	Complex granulated fertilizer containing - 18% nitrogen (N) in the ammonium form - 46% of phosphoric anhydride (P_2O_5) soluble in water and ammonium citrate.	Complex granulated fertilizer containing - 18% nitrogen (N) in the ammonium form - 46% of phosphoric anhydride (P_2O_5) soluble in water and ammonium citrate.
Pesticides	no pesticides	no pesticides
1kg of seeds costs	20 MAD	40 MAD
Plant yield per hectare	1TON / hectare	3 TON / hectare
Yield of resin per 100kg inflorescences	1.5kg to 2kg	4kg to 5kg
Cost of 1kg of premium resin	4000 MAD to 6000 MAD	6000 MAD to 7000 MAD

MATERIALS AND METHODS

Plant material

The whole plant of cannabis is collected in December 2014 with its roots, leaves, flowers, seeds, and stems from three sites in Taounate region (khlalfa, Ouedka and Tafrant). We separate the parts of the plant then the leaves and bracts were crushed.

Table 2. Botanical study of two chemotypes of Cannabis

	Khardala	Kif
Roots	swivel	swivel
Stem :		
- Shape	- Polygonal, splined	- Polygonal, splined
- Color	- Green, light yellow	- Green, light yellow
- Branching	- Alternate, opposite decussate	-Alternate, opposite decussate
- Nodes (space)	- 8 – 16cm	- 5 – 13cm
-Section (solid, hollow diameter)	- hollow, circular , 4mm	- hollow, circular, 4mm
Leaves :		
- Disposition (alternate, opposite)	- petiolate	- Petiolate
- Compounds	- Alternate	- Alternate
- Limbe (whole, toothed)	- Size of limbe: 13,5- 18,5cm	- Size of limbe: 6,8- 14,6cm
- Nervation	- Siz of petiole: 5,7- 14cm	- Size of petiole: 1,5- 4,9cm
- Particularity	- 9 folioles	- 7 folioles
	- toothed (very pronounced)	- toothed
	- Pinnate	- Pinnate
	- thin fibers	- thin fibers
Fruit :		
- Shape	- Oval (pointed at top)	- Oval (pointed at top)
- Size	- 0,4 cm	- 0,4 cm
- Color	- light brown to dark	- light brown
	- Spotted (dark brown spots)	

Extract Preparation

Extraction was carried out in an ultrasonic bath. Flasks containing 20 g of air-dried and crushed plant material and 200 ml of ethanol were immersed in the ultrasonic bath. Sonication was performed with ultrasound frequency 35 KHz, for 45 min. After filtration each mixture was evaporated under vacuum to obtain crude extracts.

Botanical study of Cannabis

The study is performed at the time of harvest in the region of Khlalfa with ten feet of each chemotype, Kif and Khardala in different parts of experimental area.

Microscopic study of Cannabis

The microscopic study of the powder has a great importance in the identification of *Cannabis Sativa*. In this study, leaves and bracts are studied using a biological research microscope (MOTIC). The microscope is equipped with a light source which improves the contrast and gives a clear and visible picture of the sample. Microscopic observations of leaves at a magnification X40, X100 and X400, are performed using an external light source (Fig.5). Three drops of chloral hydrate (100mg / 60ml) are deposited on the blade, then adding a small amount of the powder, all covered by a cover slip and then drying the preparation using a lighter. The microscopic preparation thus obtained is ready to be observed through the light microscope.

Thin layer chromatographic method

A small sample (2g) of cannabis extract is soaked in 20ml methanol, with appropriate stirring for 10 minutes. The methanol solution is evaporated by a rotavapor after filtration. The residue is reconstituted with 1ml of toluene. Using a TLC plate, spots from different samples of cannabis are placed in a container with hexane-diethylether (80:20 ml). The plate is removed from hexane- diethylether container and sprayed with Godin reagent, Cannabinoids compounds appear as purple – yellow, purple – pink, purple - red to purple – gray.

RESULTS

Botanical study of Cannabis

The botanical study of Khardala and kif has demonstrated a significant difference in the stems, leaves and fruits.

Microscopic study of Cannabis

The observation by optical microscopy of the powder of the two chemotypes of *Cannabis sativa* has shown the characteristic elements of this species by the presence of cystolithic/acystolithic unicellular hairs, multicellular glandular trichomes, calcium oxalate druses as well as anomocytic stomata. The observation of leaves has shown no significant difference between Kif and Khardala.

Thin layer chromatographic method

Although TLC does not demonstrate good accuracy as the GC-MS or HPLC, it allows to perform an approximate measurement of cannabinoids quantity in a sample, as well as to check their presence or absence. The analysis of the chromatograms has shown the presence of 11 compounds of cannabinoids for Kif and 10 compounds for Khardala. The calculated Rf revealed the presence of acid forms (CBDA, CBCA and THCA) with a slight dominance of THCA in Khardala than in Kif. For the free forms (CBN, CBD, THC, THV and CBC), we don't note the presence of THC in the two chemotypes in the three sites with an interesting amount of CBC in the Khardala of Tafrant (Figure 24).

DISCUSSION

The botanical study of Kif and Khardala has shown that they are two chemotypes that are greatly different morphologically. Kif is a variety of medium size (1.5m), with yellow-green, less loaded branches. The seeds are light brown in color. Khardala is larger (2m), having more loaded branches, yellow green, the number of leaflets of their leaves is more than those of Kif. They are also characterized by a stronger smell than Kif. Their seeds are mottled brown to dark brown. Microscopic observation of the cuticle of the upper leaf (×100) has shown that is contains a lot of non-glandular hairs, while microscopic observation at the same magnification of the lower surface of the leaves revealed droplet Cannabis resin, with trichomes sharper for Kif. Microscopic study of powders showed no difference, except longer non-glandular hairs for the Kif. Morphologically Khardala is easily distinguished kif by its rich branches, stocky, laden with dense buds and heavy, highly aromatic, while Kif is much

Table 3. Values of Rf and the percentage of the corresponding cannabinoids

Khlalfa		Tafrant		Oudka	
Khardala	Kif	Khardala	Kif	Khardala	Kif
Rf1 : 0.02 (NI) 7.54% purple - yellow	Rf1 :0.02 (NI) 7.14% purple - yellow	Rf1 : 0.02 (NI) 7.14% purple - yellow	Rf1 : 0.02 (NI) 7.14% purple - yellow	Rf1 : 0.02 (NI) 7.14% purple - yellow	Rf1 : 0.02 (NI) 7.14% purple - yellow
Rf2 :0.11 (CBDA) 11.32% purple - pink	Rf2 : 0.05 (NI) 5.35% purple - red	Rf2 : 0.11(CBDA) 10,71% purple - pink	Rf2 : 0.05 (NI) 5.35% purple - red	Rf2 : 0.11(CBDA) 10.71% purple - pink	Rf2 : 0.05 (NI) 5.35% purple - red
Rf3 : 0.15 (CBCA) 7.54% purple - gray	Rf3 : 0.11 (CBDA) 10.71% purple - pink	Rf3 : 0.15 (CBCA) 7.14% purple - gray	Rf3 : 0.11 (CBDA) 10.71% purple - pink	Rf3 : 0.15 (CBCA) 10.71% purple - gray	Rf3 : 0.11(CBDA) 10.71% purple - pink
Rf4 :0.22 (THCA) 24.52% purple	Rf4 : 0.15 (CBCA) 7.14% purple - gray	Rf4 :0.22 (THCA) 23.21% purple	Rf4 : 0.15 (CBCA) 7.14% purple - gray	Rf4 :0.22 (THCA) 23.21% purple	Rf4 : 0.15 (CBCA) 7.14% purple - gray
Rf5 :0.27 (NI) 7.54% purple - gray	Rf5 :0.20 (THCA) 21.42% purple	Rf 5 :0.27 (NI) 7.14% purple - gray	Rf5 :0.20 (THCA) 21.42% purple	Rf 5 :0.27 (NI) 7.14% purple - gray	Rf5 :0.20 (THCA) 21.42% purple
Rf6 :0.40 (NI) 5.66% purple - gray	Rf 6 :0.27 (NI) 7.14% purple - gray	Rf6 :0.40 (NI) 5.35% purple - gray	Rf 6 :0.27 (NI) 7.14% purple - gray	Rf6 :0.40 (NI) 5.35% purple - gray	Rf 6 :0.27 (NI) 7.14%il faut purple - gray
Rf7 : 0.43 (CBN) 3.77% purple - gray	Rf7 :0.40 (NI) 5.35% purple - gray	Rf7 : 0.43 (CBN) 3.57% purple - gray	Rf7 :0.40 (NI) (trace) 5.35% purple - gray	Rf7 : 0.43 (CBN) 3.57% purple - gray	Rf7 :0.40 (NI) (trace) 5.35% purple - gray
Rf8 :0.48 (CBD) 11.32% purple	Rf8 : 0.43 (CBN) 3.57% purple - gray	Rf8 :0.48 (CBD) 10.71% purple	Rf8 : 0.43(CBN) 3.57% purple - gray	Rf8 :0.48 (CBD) 10.71% purple	Rf8 : 0.43 (CBN) 3.57% purple - gray
Rf9 :0.54 (THV) 11.32% purple - gray	Rf9 :0.48 (CBD) 10.71% purple	Rf9 :0.54 (THV) 10.71% purple - gray	Rf9 :0.48 (CBD) 10.71% purple	Rf9 :0.54 (THV) 10.71% purple - gray	Rf9 :0.48 (CBD) 10.71% purple
Rf10 :0.91 (CBC) 9.43% purple - gray	Rf10 :0.54 (THV) 10.71% purple	Rf10 :0.93 (CBC) 14.28% purple - gray	Rf10 :0.54 (THV) 10.71% purple	Rf10 :0.91 (CBC) 10.71% purple - gray	Rf10 :0.54 (THV) 10.71% purple
	Rf11 :0.91 (CBC) 10.71% purple - gray		Rf11 :0.91 (CBC) 10.71% purple - gray		Rf11 :0.91 (CBC) 10.71% purple - gray

NI: not identified

more slender and less rich in branches and flower buds. Microscopically, these 2 chemotypes belong to the species *Cannabis sativa*. They contain acystolithic and cystolithic unicellular hairs of different sizes, containing calcium oxalate crystals that play an essential role in the defense against herbivores. They also contain multicellular glandular hairs (glandular trichomes), with big and small sizes, secreting Cannabis resin (hashish). The stomata are anomocytic. The cells contain calcium oxalate druses crystals. There is not a marked difference between the two chemotypes. Chemically, the analysis of the chromatograms has shown the presence of 11 compounds of cannabinoids for Kif and 10 compounds for Khardala. The calculated Rf revealed the presence of acid forms (CBDA, CBCA and THCA) with a slight dominance of THCA in Khardala than in Kif. For the free forms (CBN, CBD, THV, THC and CBC), we do not note the presence of THC in the two chemotypes in the three sites. However, the result revealed an interesting amount of CBC in the Khardala of Tafrant. In this context, the work of Srivastava et al., reported that some of 483 compounds identified are unique to Cannabis. This plant contains over 300 compounds. At least 66 of these are cannabinoids, five important cannabinoids found in the cannabis are Tetrahydrocannabinol (THC), Cannabidiol (CBD), Cannabinol (CBN), β-caryophyllene and Cannabigerol[8].

Figure 6. Glandular Hair : ×100

Figure 7. Glandular Hair ×400

Figure 8. Cystolithic hair ×100

Figure 9. Glandular Hair ×400

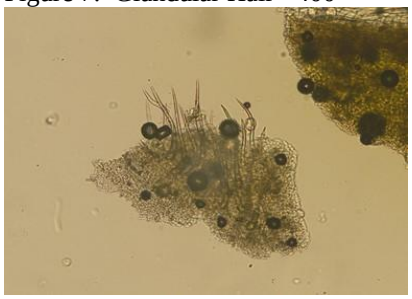


Figure 10. Fragment of bract with non-glandular hairs ×100

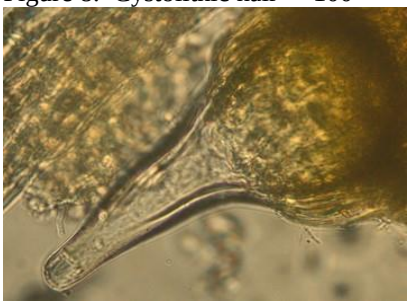


Figure 11. Small cystolithic Hair ×400

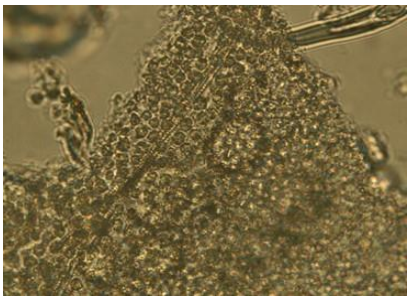


Figure 12. Cells with calcium oxalate druses ×100

Figure 13. Stigmatic papillae ×400

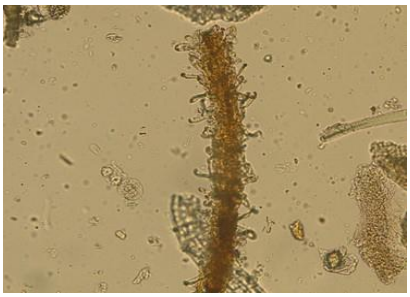


Figure 14. Stigmatic papillae ×100

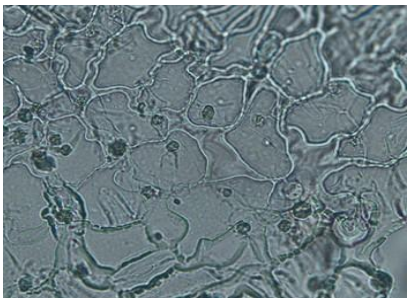


Figure 15. Cells of the epidermis with stomata anomocytic ×400

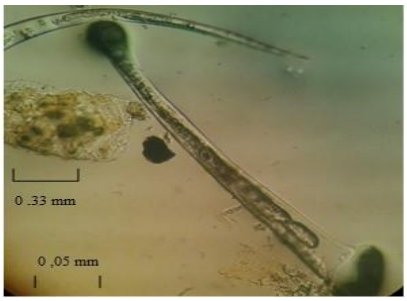


Figure 16. Long hair of Kif ×400

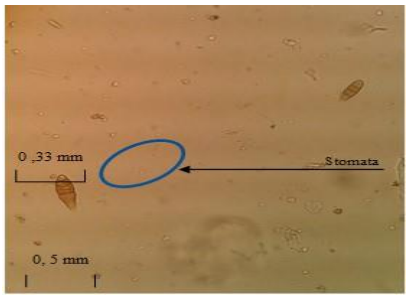


Figure 17. Small glandular hairs ×400

Figure 18. Top side of Kif leaves ×40

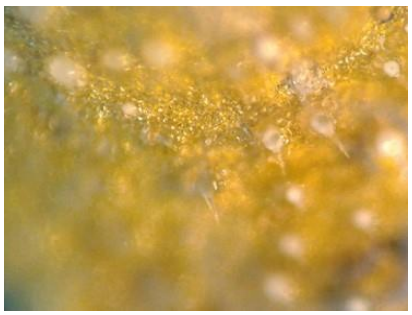


Figure 19. Top side of Kif leaves ×100

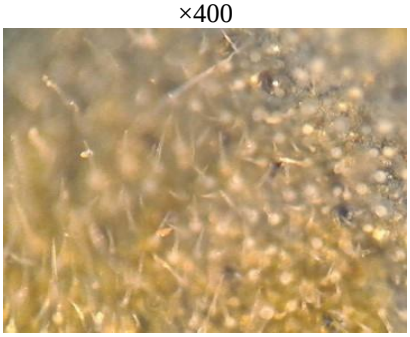


Figure 20. Underside of Kif leaves ×100

CONCLUSION

In conclusion, this is a document that will be very useful for the accurate identification of *Cannabis sativa* as it includes the botanical study, microscopy and chemical tests such as TLC as identification technique. Several other quantitative methods as GC, GC-MS, HPLC,

Figure 21. Top side of Khardala leaves ×40

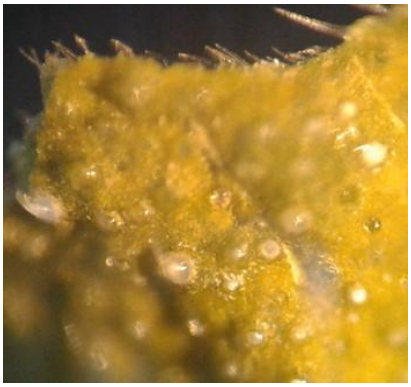


Figure 22. Top side of Khardala leaves ×100

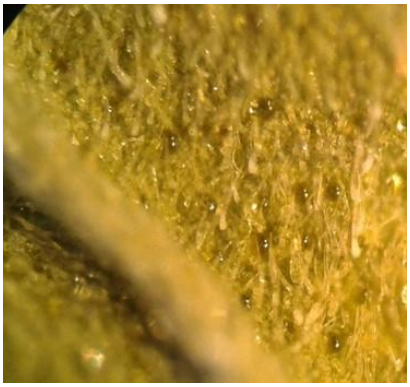
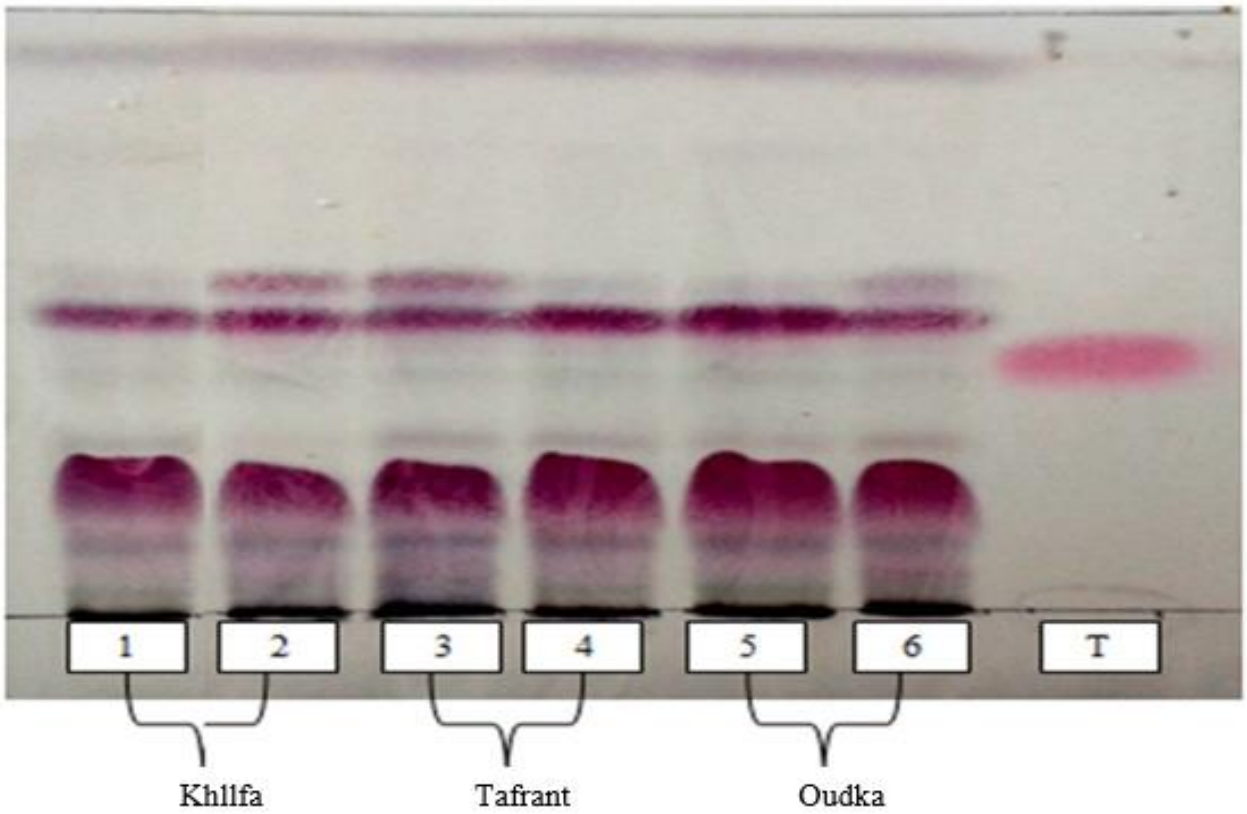


Figure 23. Underside of Khardala leaves ×100



1: Khardala of Khlalfa region; 2: Kif of Khlalfa region; 3: Kif of Tafrant region; 4: Khardala of Tafrant region; Khardala of Oudka region; 6: Kif of Oudka region; T: Thymol

Figure 24: Thin layer chromatographic of Cannabis

Spectrophotometers can also be adopted for expanded results.

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