"Finola" Cannabis Cultivation for Cannabinoids Production in Thessaloniki-Greece

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Abstract

Cannabis has garnered a great deal of new attention in the past couple of years due to the increasing hopes of its legalization for recreational use and indications for medicinal benefit. The increasing consumption and cultivation has led to a multiplication of scientific studies. Focus was placed in this study foremost on yielding morphological data (length of the plant, inflorescence fresh and dry weight) for appropriate mechanical harvest and biochemical cannabinoids analysis of the industrial cannabis "Finola" that is newly grown in Greece. The average, standard error and the coefficient of variation were estimated in case of necessity and the correlation among all results was done using Microsoft Excel 2010 and Minitab 19 Software. Furthermore, three chemical analyses for TLC and NMR techniques were applied for analysis. The Cannabinoid quality or chemotype analysis was also calculated. After extraction and isolation of cannabinoids using ethanol and other separation compounds, cannabinoid acids, tetrahydrocannabinol (THC), cannabidiol (CBD) and some other cannabinoids were extracted, isolated, identified and isolated with no delays or limitations. Finola cannabis provided a scientific background that may be considered by the Lebanese growers to accelerate and improve the relative mentality and to provide a collection of relevant scientific information, upon which the field of cannabis analysis can continue to grow.

Keywords: Finola, hemp, cannabinoids, cannabidiol, nuclear magnetic resonance

1. Introduction

Cannabis belongs to the family Cannabaceae. It originated thousands of years ago in Asia and has been spreading into many regions of the world, eventually to the Americas and the rest of the world (Warf, 2014). The word "cannabis" is used in various ways. In its broadest sense, it refers to the cannabis plant (*Cannabis sativa* L.), especially its psychoactive chemicals (employed particularly as recreational and medicinal drugs), fiber products (such as textiles, plastics and lots of construction materials), edible seed products (now in over a hundred processed foods) and all associated considerations (Chandra et al., 2017). *Cannabis* is considered as a chemically complex species based on its numerous natural constituents. It contains a unique class of terpenophenolic compounds called cannabinoids, which have been extensively studied since the discovery of the chemical structure of tetrahydrocannabinol commonly known as THC, the main constituent responsible for the psychoactive effects. In *Cannabis sativa*, cannabinoids are present in all parts of the plants, but the highest concentration can be found in glandular trichomes on the surfaces of leaves and flowers (Hemphill et al., 2004) and their detection is of great concern. Several analytical methods for identification can be used including immunoassays (Enzyme-multiplied immunoassay technique (EMIT), enzyme-linked immunosorbent assay, fluorescence polarization, and radioimmunoassay), planar chromatography techniques, classical thin-layer chromatography (TLC), Nuclear Magnetic Resonance (NMR), optimum performance laminar chromatography (OPLC), and automated multiple development (AMD), gas chromatography-mass spectrometry (GC-MS), and high-performance liquid chromatography (HPLC) (Galand et al., 2004).

Finola is an oilseed hemp variety that was developed in Finland. The European Union (EU) Plant Variety Rights were granted for the Finola variety in 1999, while it was already accepted onto the Canadian list of hemp

cultivars in 1998, where it has remained to this day. After additional delays in Europe, Finola was eventually admitted to the EU list of hemp cultivars, after a special category for oilseed hemp was created in November of 2003. Before that time, only fiber hemp varieties were recognized as hemp in the EU. Finola was the first industrial hemp variety to be registered as an oilseed crop. Finola has been cultivated in the United States since 2014 (Callaway, 2014). Several countries such as Canada, Denmark and the United States approved several cannabinoids for medical applications, particularly for the management of emesis and neurological conditions. A combination of Tetrahydrocannabinol (THC) and cannabidiol (CBD) has been approved for spasticity treatment in 25 countries. In Lebanon, according to Law 673, it was illegal to harvest, produce, trade, or possess illicit drugs- including Cannabis. Exceptions include issuing special cultivation licenses by the council of ministers for academic or public institutions for scientific or medical research purposes (article 12 of Law 673). Special permissions are also issued for individual drug use through medical prescriptions. The implementation of the law remains a significant challenge as cannabis use is still widespread among the Lebanese populations and cannabis is still cultivated, mainly in the Bekaa' valley, paving the way to illegal drug trade and smuggling. Taking into consideration that legalizing the cultivation of cannabis for medicinal purposes from April 2020 and moving it from an illicit to a legal market can release up to $1 billion in revenue for the government according to Mackenzie's estimation. The aim of this study was to confirm the morphological properties in the field for Finola cannabis strain, to prove its mechanical harvest at the appropriate time, to study the cannabinoids extraction, separation, isolation and identification, to study beneficial uses of the analyzed compounds that may be for agricultural (food and industry) or pharmaceutical (drugs) uses, to deliver data for the Lebanese society related to the best techniques used abroad in growing Finola cannabis strain that may help its regulation after legalization.

2. Material and Methods

Outdoor plant cultivation, sampling and grain moisture content at harvest period techniques are applied according to The Hemp Way Company's protocol. "Hemp Way" is a company, located in Thessaloniki-Greece, specialized in retailing high quality cannabis seeds by combines traditional techniques and new technologies and innovating in cultivation, processing and storing of cannabis and its by-products. The Hemp Way Company aim is to control all production stages, cultivation, processing and packaging, from the purest raw materials to innovating high quality products.

2.1 Outdoor Plant Cultivation

The outdoor cultivation of Finola strain was preceded by a perennial breaking crop, such as clover or green manure plow down, with 15-20% additional nitrogen added urine as manure in order to increase nutrient availability for rapid initial growth and to reduce any weed pressure. Finola was sowed in 1 cm warm, moist, weed free, well-drained sandy loam soils (above 15 °C and pH near 6.5) rich in organic matter with high nutrient availability and mycorrhizal symbioses, in May 2019 in fields related to Way hemp company in Thessaloniki Greece, following good soil, farming experience and proper nutrient levels essential for successful organic oilseed hemp production. The Humidity was around 75% during the juvenile stage and about 55% during the active vegetative and flowering stages and the temperature was about 25 to 30 °C. Donum plots were grown in a planting growth cycle of one week difference.

2.2 Plant Sampling in the Field

Finola is a rapidly maturing variety of hemp, which requires some vigilance to recognize the correct sampling time. Sampling begun 10 days after the onset of flowering, which was about 30-40 days after sowing Finola. Flowering ended near 50 days after sowing. The latest possible sampling time begun 10 days after the end of flowering (about 60 days after sowing). Thus, field sampling may begin 40-50 days after sowing, and the latest possible sampling time can be no later than 60 days after sowing. Cannabidiol content increases with the age of plant, reaching the highest level at the budding stage and achieve a plateau before the onset of senescence. The maturity of the crop is determined visually and confirmed based on the CBD and other cannabinoids content in samples collected at different growth stages of the plants. Sampling times may begin earlier if hot and dry conditions occur because late field samples may sometimes result in THC levels over 0.2%. Since the whole plant does not mature at the same time, mature upper buds are harvested first and other branches are given more time to achieve their maturity. Field cultivated Cannabis plants are generally bigger and contain higher biomass than other plants grown differently.

2.3 Grain Moisture Content at Harvest Period, Storage and Laboratory Sampling

Approximately 100-130 days after sowing, the top third of the crop was combined for grain while the plants were still "green" (70-90% seed head maturity). Harvesting when the crop is partially green helps to minimize cutting and wrapping problems. Dry field conditions are essential for a good harvest. Mature grain can be

harvested after 120 days in Mediterranean climates whereof birds were noticed in the field. Grain moisture content was tested with a calibrated meter to be at least 10-15% at harvest period and grain moisture should be kept less than 9% after cutting in order to prevent seed heating, reduce mold growth and to preserve seed quality by simple aeration.

The mature grain was harvested with a straight cut header grain combine to chop the remaining stalk and return the harvested biomass to the field. A threshing drum and straw chopper worked surprisingly well for this crop and some minor modifications were made to limit fiber wrapping and to speed up harvesting. Modest ground speeds and input rates, with initial high engine speeds were applied to limit mechanical problems (wrapping problems and subsequent fire hazard). Checks were also made for the front drum, breather, sprockets, feeder chain, drive axle, grain elevator axle heads and any other moving parts, inside and outside the combine. Also, the feeder house was removed, checked and unblocked every 2 hectares.

Finola grain drying facilities was accomplished by transferring the grains to heated trailer beds with ventilation at low temperatures (30-40 °C max) and high volumes of airflow, for 10-14 days. Finola grain cleaning was done with 1.60-3.25 mm oblong and 2.50-5.00 mm round sieve sizes respectively. Dried grain seeds were stored in bins or 500 kg tote bags, away from birds and can be stored for more than two years.

The samples used for this study have been submitted for the analysis to the Laboratory of Pharmacognosy, School of Pharmacy at Aristotle University of Thessaloniki-Greece, by The Hemp Way Company. They were packed (100 grams bags), numbered (from one to thirty) and labeled (Finola). Field inspection was realized for every stage of growth and development in May 2019 since plots were grown in a planting growth cycle of one week difference. Thirsty plant samples have been randomly selected for morphological studies related to crop yields only at harvest time, in September, 2019. The length of the plant, the fresh and the dry weight of the inflorescence were studied. The fresh and dry weight inflorescence average, standard error and the coefficient of variation were estimated. The correlation among all results was done using Microsoft Excel 2010 and Minitab 19 Software.

Morphological characteristics of Finola plants were studied and related measures of variability (minimum, maximum, median, standard deviation, standard error and coefficient of variation) were estimated using Microsoft Excel 2010 and Minitab 19 Software.

2.4 Analysis

Instrumental methods were used for the identification, classification and individualization of cannabinoids in cannabis plant extracts. The botanical identification of *Cannabis sativa* L. plant specimens consists of physical examination of the intact plant morphology and habit, followed by the microscopy examination of leaves for the presence of cystolith hairs. The very abundant trichomes, which are present on the surface of the fruiting and flowering tops of cannabis, are the most characteristic features to be found in the microscopic examination of cannabis products (Bruni et al., 1983). Because of the complex chemistry of cannabis, separation techniques, such as GC or liquid chromatography, often coupled with MS, are necessary for the acquisition of the typical chemical profiles and the sensitive, specific, qualitative, and/or quantitative determination of cannabis constituents. However, especially for screening purposes and on-site field testing, non-instrumental techniques like thin-layer chromatography (TLC) and color reactions were applied (Brenneisen, 2007)

2.5 Thin Layer Chromatography

Ten grams of dried Finola strain plant materials were kept at -20 °C along with the ethanol bottles for 24 hours. They were grinded and subsequently soaked, sonicated and filtered for two more times. The filtrate was poured and diluted (1:2). Half of the filtrate volume was directly condensed in the bubble of the Rota vapor and to the other half; a binding compound was added to remove the chlorophyll before the condensation. The condensed extract materials were and kept at -20 °C for further analysis.

Cannabinoids were separated on thin-layer chromatography (TLC) Si $60F_{254}$ plates stationary phase. Samples were applied on TLC plates as six spots of minimum size with a homogeneous distribution of material on one cm line of its starting zone using a glass capillary. Four developing solvents were eluted. The plates were put in a chamber containing 50 ml of the following eluents: dichloro-methane/methanol (98:2 v/v), DM (100%), hexane-ethyl acetate (85:15 v/v) and acetone/dichloro-methane/tert-butyl methylether/hexane (4:4:12:80 v/v). Plates were put under U.V light, poured with vanillin and separated compounds of interested were marked and subjected to NMR analysis for identification.

2.6 Cannabidiol Content

Cannabidiol content increases with the age of plant, reaching the highest level at the budding stage and achieve a plateau before the onset of senescence (Chandra et al., 2009). The maturity of the crop is determined visually and confirmed based on the CBD and other cannabinoids content in samples collected at different growth stages of the plants (Burgel, 2020). Since the whole plant does not mature at the same time, mature upper buds are harvested first and other branches are given more time to achieve their maturity. Field cultivated Finola Cannabis plants were generally harvested using combines at 1.76 cm height.

2.6 Nuclear Magnetic Resonance

^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Bruker model AV 400 FT-NMR spectrometer (Karlsruhe, Germany) with reference to TMS as standard. EI-MS spectra were obtained using a Finnigan MAT 700 instrument (San Jose, CA, USA). The ^{1}H NMR spectra (500 MHz) and ^{13}C NMR spectra (125.0 MHz) were recorded in $CDCl_3$ using AGILENT DD2 500 spectrometer. Chemical shifts are reported in δ ppm values relative to TMS (Brenneisen, 2007).

3. Results and discussion

3.1 Morphological Characteristics at Harvest Time

The samples of Finola in the field were erect, annual, dioecious, growing up to 1.8 m in height. The stems were green, hollow, cylindrical and longitudinally ridged. Secondary branches were opposite. Leaf arrangement varied from decussate at lower branches to alternate at terminal ones. Petioles were up to 7 cm long, cylindrical with a median groove along the upper side, and covered with non-glandular and glandular trichomes.

Petiolules were 0.5-1.5 cm long. The leaves were palmately 3-9-lobed, showing actinodromous venation and the youngest leaves were sometimes unlobed. The lobes were narrowly oblong-lanceolate, 3-20 cm long, up to 1.8 cm wide, dark green above, paler beneath, attenuate at base, caudate-acuminate at apex, and serrate along the margins. The serrations along the margins were prominent, curved and pointed towards the tips of the leaf blades. Each lobe had a primary midrib and several secondary veins at either side. Each of the secondary veins run out obliquely from the midrib and entered into a serration of the margin. The veins were prominently raised forming ridges on the abaxial side whereas they were impressed on the adaxial side forming grooves. The lowest pair of lobes was usually much smaller than the others and pointing backwards. In seedlings, the first pair of leaves was 1-foliolate and the second and third pairs were three and five-foliolates, respectively.

Male flowers were pale green, borne on axillary laxly branched cymose panicles. Flowers in the panicles occured solitarily, in clusters, or in 3-flowered cymules. Each flower consisted of five tepals, five stamens and a slender pedicel. The tepals were ovate-oblong, 2-4 cm long, yellowish- or whitish-green, spreading, and minutely hairy. The stamens were drooping and consist of slender filaments and oblong, greenish anthers. Pollen grains were liberated through terminal pores in the anthers.

Female flowers were dark green, subsessile and were borne in pairs. The flowers were closely aggregated at the apex of short spike inflorescences, which were densely formed in the upper axils of branches. Each flower consisted of ovary with a style that ends in a pair of long slender feathery stigmas at apex, a membranous perianth surrounding the ovary, and a bract. The style-stigma portion of the pistil in wild-growing plants generally measured about 3 mm long and the styles were usually 2-branched.

The achene fruit was ovoid, ellipsoid or subglobose, about 4-6 mm long and 3-4 mm in diameter, smooth, somewhat compressed, brownish grey and mottled, containing a single seed with a hard shell. Sometimes, the cannabis "seed" of commerce was actually the enclosed fruit in its hooded floral bract.

Male and female flowers occur in separate plants; they generally bloom during July-August. Male plants are usually taller and the female plants are usually more robust than male plants. Several cultivars with varying features occur in cultivation.

Morphological characteristics of Finola studied plants confirmed the data recuperated in the year 2018 with very small differences that may be influenced by the seed strain as well as by environmental factors such as soil type, light, water, nutrients and space (Chandra et al., 2017). Among the apparent modifications we cite in the table below (Table 1), the average length of thirty Finola plant sampels selected randomly at harvesting time (in meter), their average weight of inflorescence (gram fresh and dry weight) and related measures of variability (minimum, maximum, median, standard deviation, standard error and coefficient of variation).

Table 1. Measures of variability of yield morphological characteristics from 30 Finola strain samples (length, fresh and dry weight)

Measures of variability	Length (m)	Fresh Weight (g)	Dry Weight (g)
Average±Standard Deviation	1.760667±0.043	192.6667±12.399	37.96667±5.724
Minimum	1.7	174	26
Maximum	1.86	215	47
Median	1.75	195.5	38
Standard Error	0.007	2.263	1.045
Coefficient of Variation	0.024	0.064	0.150

The average length of studied Finola strains at harvest is 1.76±0.043 m. Also, their average fresh and dry weight inflorescences±standard deviation is about 192.666±12.399 g and 37.966±5.724 g respectively. There is no correlation between obtained results (length, fresh weight and dry weight) at $p = 0.05$ (Table 1). The standard error and the coefficient of variation show that the dispersion of the variables among studied plants is very low. The Canadian Food Inspection Agency reported in 2003 that the average Finola length in its trial is 1.13±0.136 m. Results of the Agency were supported by the official technical examination report purchased from the Plant Breeders' Rights Office in the Netherlands. Difference in length is maybe due to the density of seeds sowed in the field. Increasing sowing density causes significant shortening of plants, regardless of the date of harvest (Burczyk et al., 2009).

3.2 Thin Layer Chromatography

Samples were manually spotted on reversed phase (C_{18}) silica gel plates F_{254} and developed in saturated chambers. The separation of cannabinoids by means of this technique is not easy, because these derivatives possess chemical structures with very close substitutes. Besides, the molecular weight of cannabinoids is also very close (like THC and cannabinol (CBN) with similar molecular weight (g) of 314.47 which is close to CBD's of 310.44). Different eluents were tested:

- Dichloromethane (100%) (Figure 1);

- Dichloromethane:Methanol (98%:2%) (Figure 2);

- Hexane:Ethylacetate (85%:15%) (Figure 3);

- Acetone:Dichloromethane:Tert-Butyl-methylether:Hexane (4%:4%:12%:80%) (Figure 4).

All plates were compared with reference compounds and literature data from previous laboratory researches. It was noticed that the plates presenting the samples treated with the separatory compound added before filtration were clearer than those not treated with the same separatory compound, and this was due to its capability to capture all non-cannabinoid compounds. TLC is a low-cost method for cannabinoid analysis and approved by the United Nation Office on Drugs and Crimes (UNODC) for Cannabis routine control of cannabinoid content and of Cannabis origin (UNODC, 2009). The elution of stains using different solvent mixtures showed very similar plates and this is due to the clarity of the isolated cannabinoid compounds of interest. In our Layer Chromatography, polarity decreases in the direction of travel of the solvent or eluent (Figure 5), so from the origin of the plate in which samples were spotted, to the top of the plate. Similar results were obtained by Bele et al. (2011). The most polar were the cannabinoid acids, followed by CBN, THC, CBD and eventually CBC. Stains and polarity were confirmed by NMR analysis.
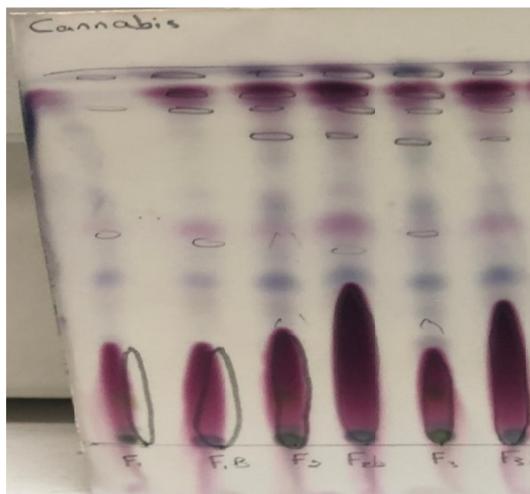
Figure 1. TLC plate with Dichloromethane (100%) solvent
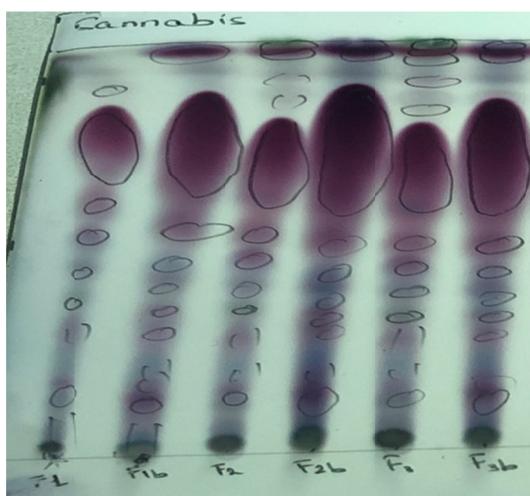

Figure 2. TLC plate with Dichloromethane:Methanol (98%:2%) solvent
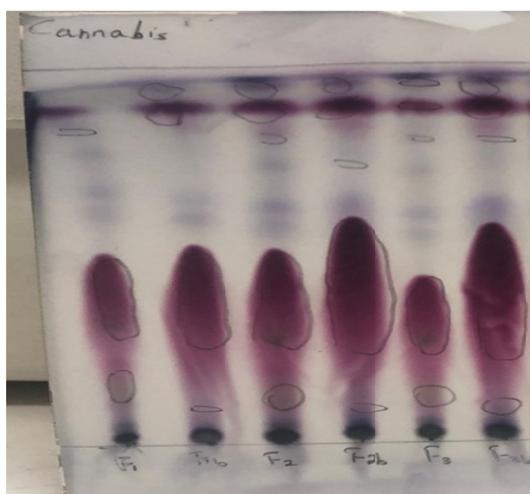

Figure 3. TLC plate with Hexane:Ethylacetate (85%:15%) solvent
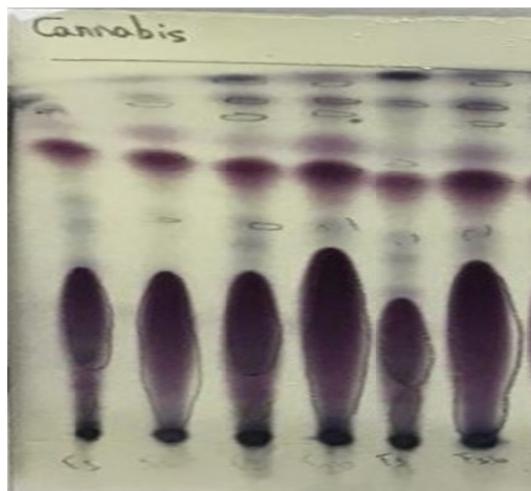
Figure 4. TLC plate with Acetone:Dichloromethane:Tert-Butyl-methylether:Hexane (4%:4%:12%:80%) solvent
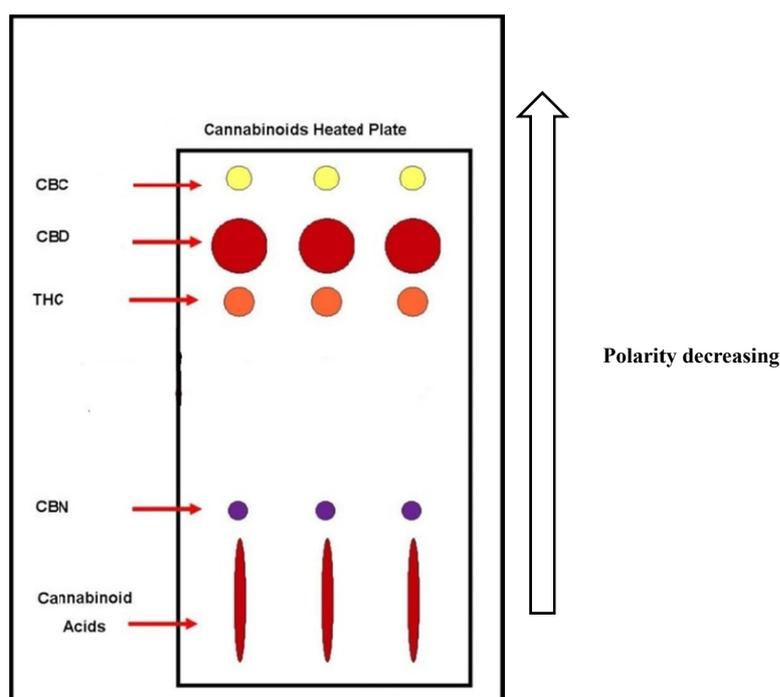
Figure 5. TLC plate representative of cannabinoids positions

3.3 NMR Spectroscopy

3.3.1 Isolation of Cannabinoids

Isolation of cannabinoids was done using a separation compound to seize all chemicals excluding cannabinoids of interest and was confirmed by TLC and NMR analysis. The 1H[1] NMR spectra (500 MHz) and 13C NMR spectra (125.0 MHz) were recorded in $CDCl_3$ using AGILENT DD2 500 spectrometer. Chemical shifts are reported in δ (ppm) values relative to TMS.

3.3.2 Analysis of Spectroscopic Data

From the ^{1}H NMR spectrum, it was observed that the chemical shift (δ), region between 0 and 3 ppm, had many signals, indicating a large amount of information. However, the signals overlap, making it difficult to realize the attributions of each signal. In contrast, the δ region between 6 and 7.25 is an interval where the hydrogen signals are more distinguishable allowing for the identification of aromatic and olefinic hydrogen atoms of cannabinoids (Figure 6).
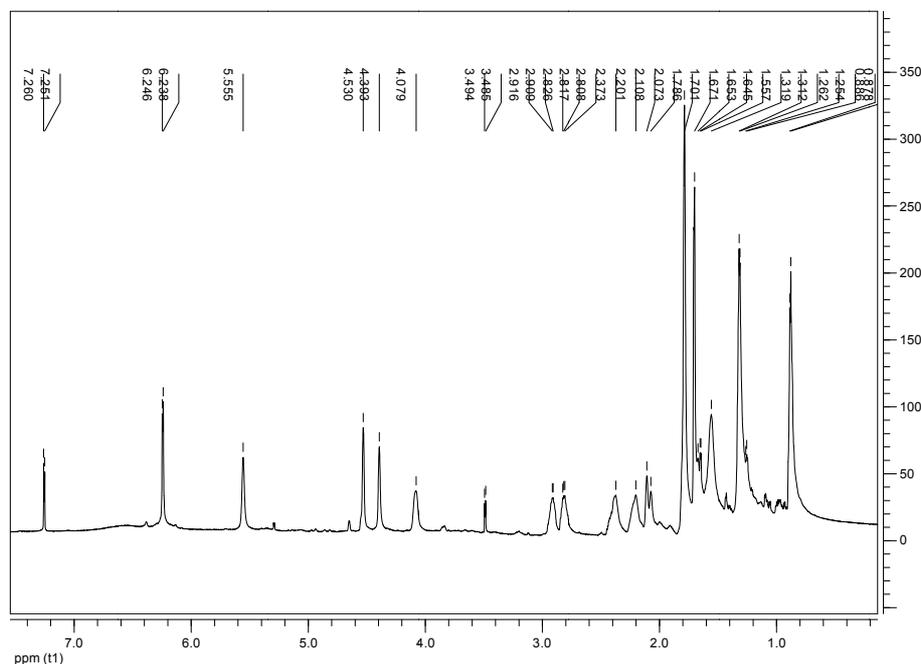
Figure 6. ^{1}H NMR (500 MHz, CDCl3) for aleatory Finola sample

From the obtained data in the spectrogram, signals were assigned to some of the compounds, allowing the determination of the major composition of the samples. Two characteristic signals appear of CBN at 7.12 ppm and 7.04 ppm, due to the aromatic hydrogens H5 and H4 respectively. Another characteristic signal appeared at 6.12 ppm, referred to as H3′ of Δ^9-THC. This reveals that Δ^9-THC was degraded to CBN, probably due to storage conditions or sample age (Figure 6).
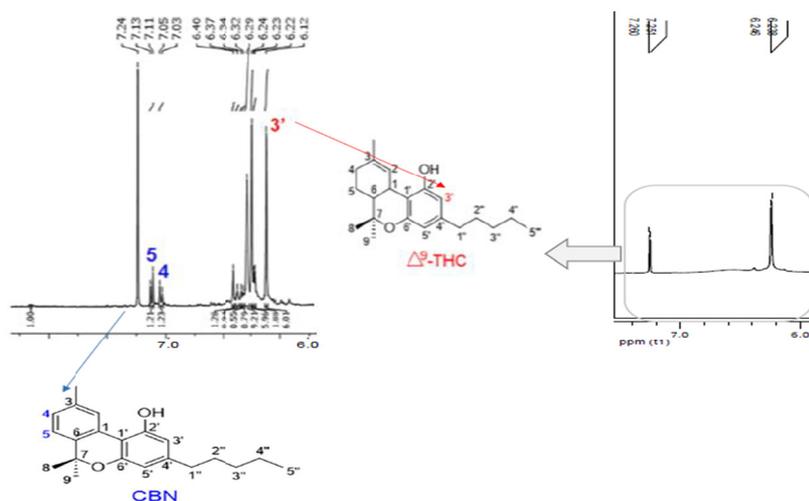
Figure 7. CBN and THC presence clarification through NMR technique

The H-9 protons show two different signals: one signal for the cis on 4.2 ppm and one for the trans configuration on 4.3 ppm, which is also known as the cis-trans isomerism (Figure 7). This reveals the place of CBD compound in the NMR spectroscopy related to the chemical structure of CBD Similar results were also obtained by Júlia et al. (2018) on 4.2-4.3 ppm (Figure 8).
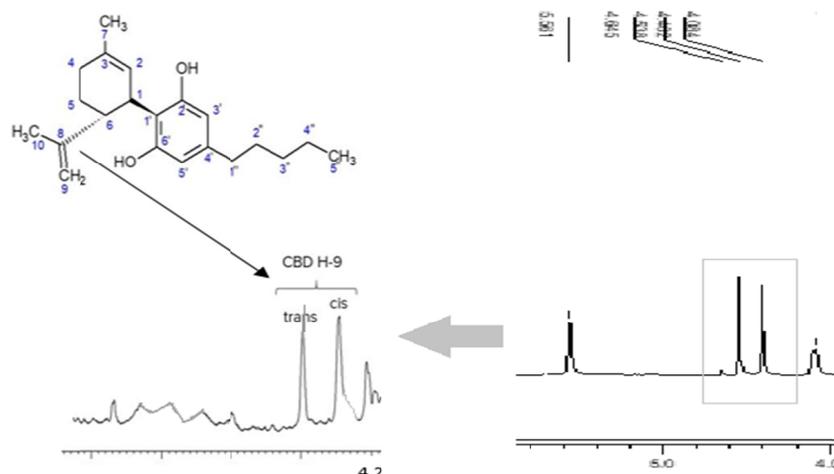
Figure 8. Double bond region showing CBD signals

NMR technique is able to clarify the presence of most principal cannabinoids present in the Finola extracts of our study, basically CBD, CBN and THC. Nevertheless, we were not capable to determine other cannabinoid compounds in Finola extracts, maybe due to its capability in restricting its cannabinoids synthesis into these analyzed compounds only. Finally it was referred that the percentage of CBD and THC in our Finola strain was approved by GC-FID in the Central laboratory of Pharmaceutical compounds by Benaki Phytopathological Institute in Greece to be 1.89 and 0.06 respectively in September 2019.

4. Conclusions and Recommendations

This work explained the methods of Finola strain (*Cannabis sativa* L.) growth and morphological traits used for hemp harvest at the appropriate time in order to extract, isolate and identify cannabinoids from panicle samples.

Extraction and isolation were done using ethanol and oil was obtained from the samples. Oil was examined by TLC and NMR spectroscopy for analysis using TLC plates and NMR spectroscopy graphs. With TLC, it was possible to detect the presence of cannabinoid acids, Cannabidiol (CBD), Tetrahydrocannabinol (THC) and Cannabinol (CBN) when compared with other TLCs but this method did not offer their purification. The best TLC plate obtained was the one containing the mixture (Acetone:Dichloromethane:Tert-Butyl-methylether: Hexane) = (4%:4%:12%:80%), when compared to others which gave fair good results. NMR spectroscopy confirmed the presence of CBD, THC and CBN in the analyzed samples through peaks present in the graph and position of hydrogen molecules in the cannabinoid structures. Cannabis is accepted as a medicinal plant due to the impressive amount of therapeutic and pharmacological properties of cannabinoids.

In Lebanon, Legalization of cannabis has recently taken place and its regulation has to be settled the sooner the better especially when having on hand previous results of specific trials of cultivating Finola in addition to other strains. Specific facilities, protocols and analytical methods of identification and quantification of cannabinoids should be executed so that all the cultivation and production of drugs could be perfectly controlled from the field to the manufacturing companies.

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