Journal of Agricultural Science; Vol. 12, No. 7; 2020
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Yielding Morphological Characteristics and Biochemical Analysis of "Karma Lemon" Cannabis Producing Cannabinoids in Thessaloniki-Greece

Dani Fadel[1], Najoie Assaad[2], Nour Wahab[1] & Diamanto Lazari[3]

[1] Department of Languages and Literature, Faculty of Pedagogy, Lebanese University, New Rawda, Lebanon

[2] Department of Plant Production, Faculty of Agriculture, Lebanese University, Dekwaneh, Lebanon

[3] School of Pharmacy, Department of Pharmacognosy, Aristotle University of Thessaloniki, Thessaloniki, Greece

Correspondance: Najoie Assaad, Department of Languages and Literature, Faculty of Pedagogy, Branch 2, Lebanese University, New Rawda, Lebanon. Tel: 961-168-0193. E-mail: najoie.assaad@outlook.com

Received: May 9, 2020 Accepted: June 8, 2020 Online Published: June 15, 2020

doi:10.5539/jas.v12n7p182 URL: https://doi.org/10.5539/jas.v12n7p182

Abstract

Cannabis has been widely used by humans over many centuries as a source of fiber, oil and for medicinal purposes. Its use was illicit in numerous countries, including Greece and Lebanon. "Karma Lemon", one of the newest cannabis strain originated from Italy, is selected in this study to analyze its components using various techniques starting from the extraction, isolation and identification of cannabinoids using separatory compounds and NMR techniques as well as the main important morphological traits of this strain to be harvested at an appropriate time for medicinal uses in Greece and later on, in Lebanon. Thirty different samples were selected from the field of respected "Hemp Way Company" in Thessaloniki and studied for morphological traits. These were related to the length of the plant at harvest time (1.809 m) needed for the use of combines and the weight of inflorescence (213.5 g fresh and 40.8 g dry) for oil or seed production. Three samples of Karma Lemon cannabis strain inflorescence were analyzed at the Laboratory of Pharmacognosy, School of Pharmacy, in the Aristotle University of Thessaloniki in Greece, after proper extraction and isolation using ethanol and other separation compounds. TLC and NMR techniques were used to visualize and identify cannabinoids present after isolation. Cannabinoid acids, CBG, CBN, THC, CBD and other cannabinoids were identified and isolated.

Keywords: Karma Lemon, thin layer chromatography, nuclear magnetic resonance, cannabinoids

1. Introduction

The genus "*Cannabis*" contains some of the oldest plants used for food, fiber, and medicine. *Cannabis indica* belongs to the family Cannabaceae. Its earliest cultivation originated in central Asia, wherefrom the crop spread to the Middle East, China, Europe, and the Americas during the early 16th Century (Radwan et al., 2017).

The Cannabis plant and its products consist of an enormous variety of chemicals whereof some of the 483 compounds were identified and more than 60 cannabinoids were found unique to the genus, whereas the terpenes, with about 140 members forming the most abundant class, are widespread in the plant kingdom. The main biologically active cannabinoids are tetrahydrocannabinol, commonly referred to THC, known for its psychoactive properties and the nonpsychoactive cannabidiol (CBD), characteristic of fiber-type cannabis (Brenneisen, 2007). Cannabis legislation models include prohibition, legalization for medical purposes, decriminalization, and legalization of recreational use (Hilal et al., 2018). Cannabinoids and terpenes develop in the resin glands, or trichomes, on the flower and leaves of cannabis plants. Many other plants produce cannabinoids, but they are found in the highest concentration in cannabis. Terpenes and cannabinoids work together to develop a strain's particular flavor and resulting high, a phenomenon known as the entourage effect. The different compounds interact synergistically to amplify the benefits of the plant's components (Jacobs, 2019). Several countries approved the use of cannabinoids for the treatment of various medical conditions.

Cannabis cultivation is a major source of living in this impoverished area of the Mediterranean countries, where alternative crop programs have failed to meet the economic needs of farmers.

The Objectives of this study were to study and confirm the morphological traits in the field necessary for harvesting for Karma Lemon cannabis strain (length of the plant at maturity and the weight of the inflorescence) and for the use of combine machines at the appropriate time, to extract the cannabinoid content using alcohol, to separate, isolate and identify cannabinoids for pharmaceutical uses using TLC and NMR, to deliver data for the Greek and for the Lebanese society related to the best techniques used to grow and develop cannabis in similar environmental and soil conditions for medicinal and pharmaceutical uses.

2. Material and Methods

2.1 Morphological Analyses

Karma Lemon cannabis was selected to collect morphological and biochemical data that may be used for growing this strain in Lebanon as a result of climate, environment, soil, water system and other similarities with Greece in addition to the importance of Karma Lemon in terms of cannabinoids content.

The appearance of thirty plants of Karma Lemon cannabis strain were selected randomly, in a field of one dunum from thirty three hectares owned by "The Hemp Way" company in Thessaloniki (Kalamariá) in Greece (37°48′35.6″ North, 23°51′35.2″ East and has an average elevation of 50 m above sea level).

This field is divided into several sections with 13 rows exist. The main cause of the adoption of this system is to harvest Cannabis in a continuous way because these sections contain different growth stages of one week time difference from seeding to maturity. These offered samples are from the last year cultivation and they were harvested, dried and put in sealed packages.

Some traits were determined on each of these plants. The length of the plant at maturity was measured by taking the length (in meter) of Karma Lemon plant at harvesting time using a measuring tape (in cm); the height was taken from the surface of the soil to the top of the plant. This trait is important for setting the cut at an appropriate height at the harvesting time, using the combines, approximately 2 meters for Karma Lemon. It is very important that the plants have almost the same length when cultivated to reduce the loss of productivity in the field.

The weight of inflorescence (gram fresh and dry weight) for the 30 different samples was also measured on fresh and was labeled to be taken again after drying (two weeks were necessary for complete drying at room temperature). This indicates the productivity of the Cannabis. These two traits were averaged and a standard deviation was calculated. Standard error and coefficient of variation were also calculated in case the standard deviation is high at $p = 0.1$.

2.3 Chemical Analysis

Ten grams of dried Karma Lemon plant materials were kept at -20 ^{o}C along with the ethanol bottles for one overnight, 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 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 ^{o}C for further analysis.

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.

Cannabinoids were separated on thin-layer chromatography (Si $60F_{254}$ plate stationary phase TLC). 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 methyl ether/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. 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 and ^{1}H NMR spectrum with signals was observed for the identification of aromatic and olefinic hydrogen atoms of cannabinoids (Brenneisen, 2007).

3. Results and Discussion

3.1 Morphological Characteristics at Harvest Time

The samples of Karma Lemon in the field were between 1.50 and 1.90 meters length with an erect stem that is sometimes hollow. The basal leaves were opposite but the highest one was alternate, palmate, lanceolate, sharp spikes up to 10 cm long and their color was deep green with hints of yellow and brown. 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.

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.

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.

Morphological characteristics of Karma Lemon studied plants confirmed the data recuperated in the year 2018 with very small differences that may be influenced by the 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 Karma Lemon plant samples 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. Average±standard deviation of the morphological traits of studied Karma Lemon plants with shown minimum and maximum values

Morphological characteristics	Average±standard deviation	Minimum and Maximum values
Length of Karma Lemon plants (m)	1.809±0.080	1.5-1.9
Weight of fresh inflorescence (g)	213.5±4.783	205-220
Weight of dried inflorescence (g)	40.8±3.377	35-40

The average length of studied Karma Lemon strains at harvest is 1.809 ±0.080 m. The use of combine harvesters was adequate to cut hectares of crops using a sharp at 1.80 m (Table 1). The optimal length of Karma was reported to be between 1.25 m and 2.00 meters (Chandra et al., 2017).

The average fresh weight inflorescences ± standard deviation of the studied plants is about 213.5±4.783 g and (Table 1). For the weight of fresh inflorescence, the very low standard variation means the stability of yield at plants level. The optimal Karma weight average published ranged between 190 g and 235 g (Chandra et al., 2017).

Regarding the dry weight of inflorescence (40.8±3.377 g), the standard variation was a bit high and this is due to the trimming of some organs (leaves and not flowers or seeds) that may affect its weight and making the difference almost double to that of fresh weight. The optimal weight according to Chandra et al. (2017) was 30 g to 50 g.

The planting density of Karma Lemon in the fields of Hemp Way Company in Thessaloniki was about 4500 plants/dunum.

These results reveal that this plant can be harvested using combine machines because of the close length obtained of the samples and that the weight of the dry and fresh inflorescences is optimal. This reflects the good stable quality of the harvested plants and the certainty to obtain the expected cannabinoids.

3.2 Thin Layer Chromatography

Samples were manually spotted on 10 × 20 cm reversed phase (C18) silica gel plates F_{254} and developed in saturated normal chambers as explained previously (saturation time 15 minutes). TLC is a suitable method for screening different samples, but it is qualitative and only semi-quantitative test. This means that it cannot be used to accurately determine the percentage of THC, CBD or any other compound. TLC can detect if a sample has a particular cannabinoid, and the approximate ratio of a cannabinoid to another.

The separation of cannabinoids by the mean 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 CBN with similar molecular weight (g) of 314.47 which is close to CBD's of 310.44). Different eluents were tested in this study: Dichloromethane (100%), Dichloromethane:Methanol (98%:2%), Hexane:Ethylacetate (85%:15%) and Acetone:Dichloromethane:Tert-Butyl-methylether:Hexane (4%:4%:12%: 80%) as shown in the Figure 1, 2, 3, and 4 respectively.

In the four plates, the samples treated with separatory compounds before the filtration and condensation were clearer than those not treated. These results prove the capability of it to capture all non-cannabinoid compounds. All the plates obtained were added and compared with literature data and reference compounds saved in the laboratory from previous researches. TLC is usually used for its low-cost and the capability of compounds separation. The elution of stains using different solvent mixtures showed very similar plates, and this is due to the clarity of the isolated compounds of interest.

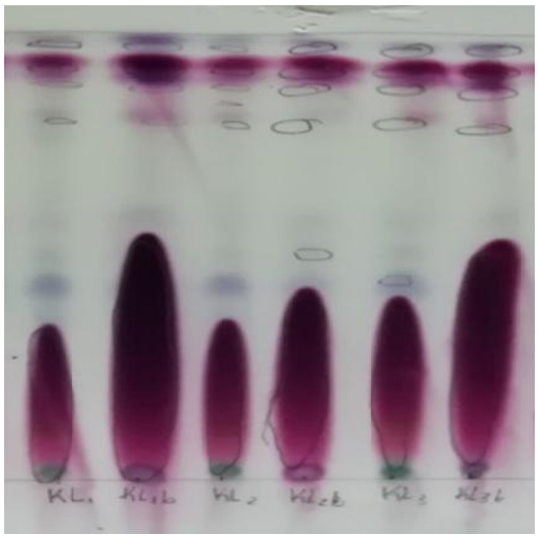

Figure 1. TLC plate with Dichloromethane: (100%) solvent

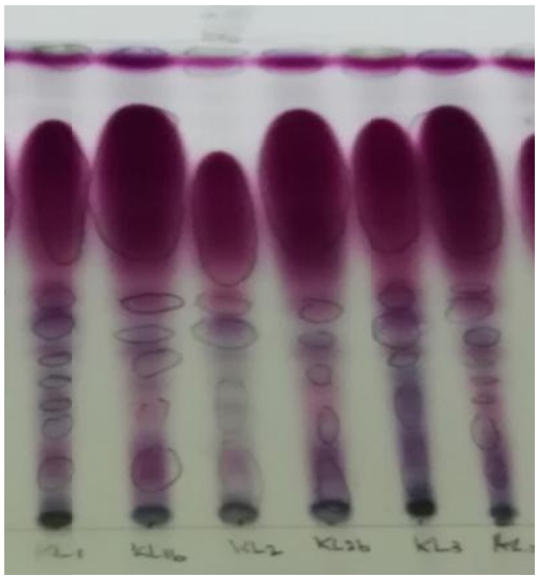

Figure 2. TLC plate with Dichloromethane:Methanol (98%:2%) solvent

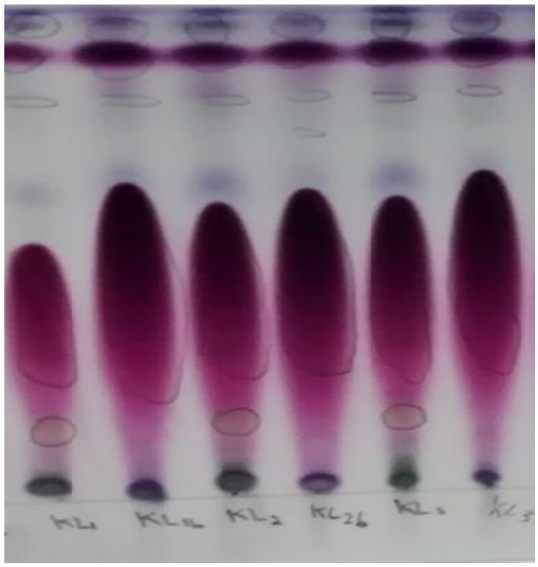

Figure 3. TLC plate with Hexane:Ethylacetate (85%:15%) solvent

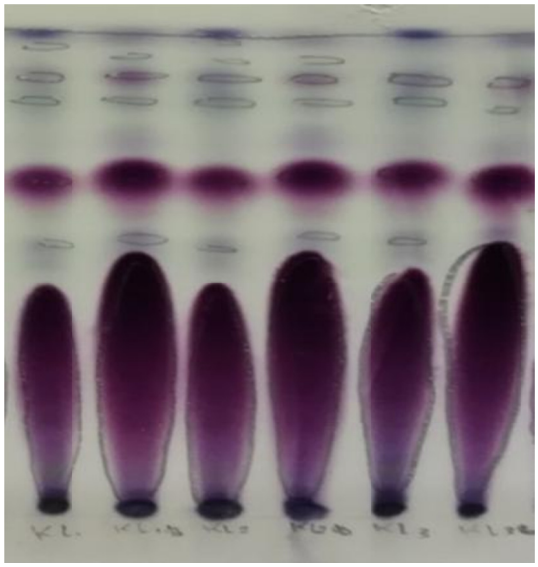

Figure 4. TLC plate with Acetone:Dichloromethane:Tert-Butyl-methylether:Hexane (4%:4%:12%:80%) solvent

This Layer Chromatography, polarity decreased 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 Iseger and Bossong (2015). The most polar were the acids cannabinoid, followed by CBN, THC, CBD and eventually CBC. Stains and polarity were confirmed by NMR analysis.

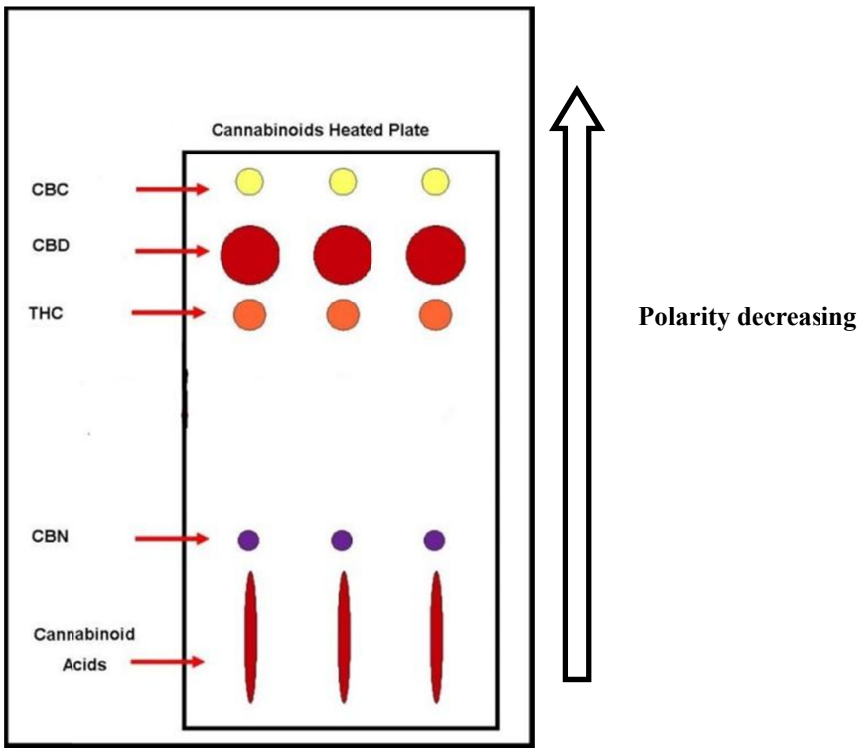

Figure 5. TLC plate representative of cannabinoids positions

3.3 NMR Spectroscopy

The spectrogram obtained by NMR shows signals that are relative to the compounds present in Karma Lemon which allow us to determine the composition of the sample.

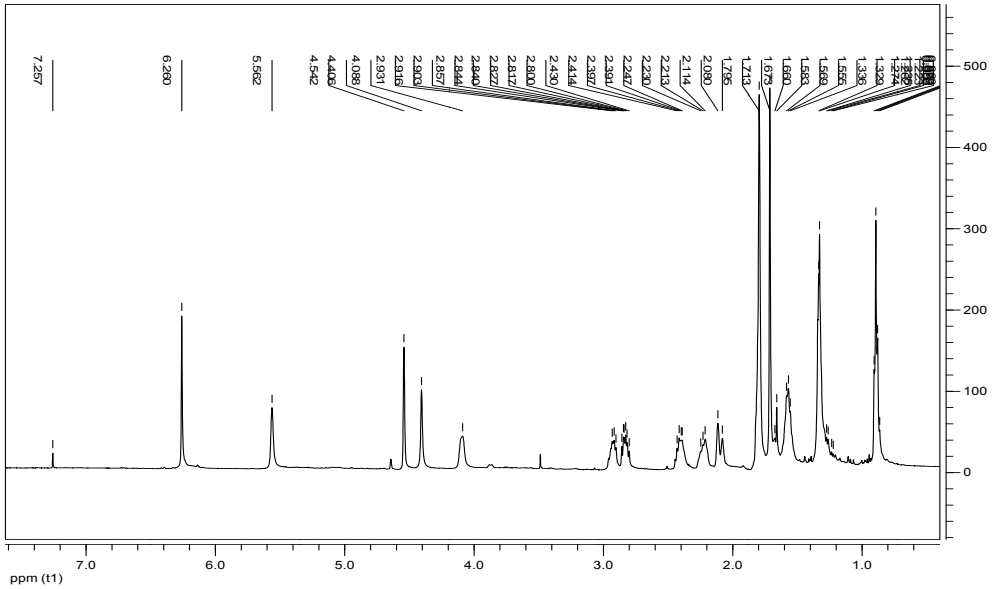

Figure 6. NMR results for Karma Lemon sample

From the ^{1}H NMR spectrum (Figure 6), it was observed that the chemical shift (δ), region between 0 and 3 ppm, had many signals, indicating a large amount of information.

Four characteristic signals appeared for CBG and CBGA at 0.85 ppm, 1.70 ppm, 1.80 ppm and 6.25 ppm due to the aromatic hydrogens H5'', H9, H7 and H5' respectively (Figure 7). The presence of CBGA indicated that some plants were not mature enough since at the beginning there was just CBGA. Then with the right conditions, CBG or one of the varied acidic precursors that precede cannabinoids were found. THCA, CBDA and CBNA all begin as CBGA. Once transformed into these acidic precursors, the chemicals go on to become medicinally potent forms like THC and CBD. In this way, CBG's acidic form gives rise to every single cannabinoid in the plant earning the name "The Mother of Cannabinoids".

Similar results of CBG were also obtained by Lewis et al (2019) on 0.8, 1.7, 1.75 and 6.25 ppm due to the aromatic hydrogens H5'', H9, H7 and H5' respectively. Moreover Pisanti et al (2017) has obtained CBG on 0.85, 1.7, 1.6 and 6.08 ppm due to H5'', H7, H9 and H5' respectively.

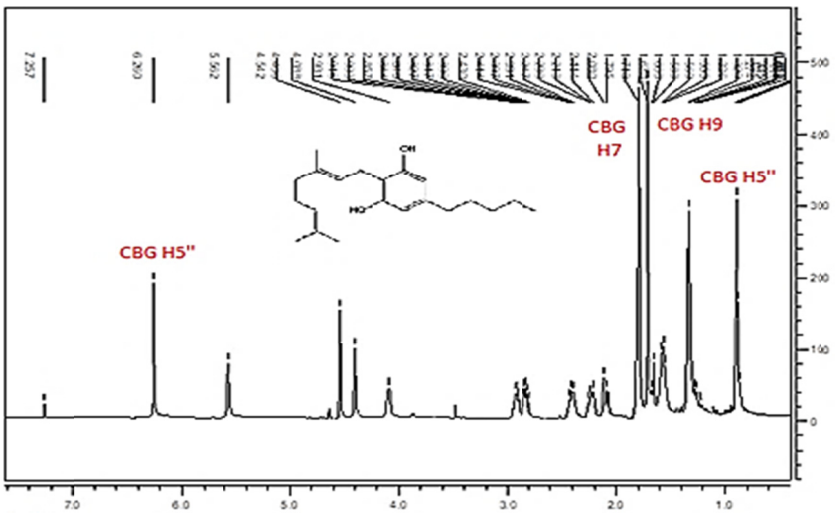

Figure 7. CBGA and CBG presence clarification through NMR technique

Another two characteristic signals of CBN appeared at 7.11 ppm and 7.05 ppm (Figure 8), due to the aromatic hydrogens H5 and H4 respectively. Other results of CBN signals were obtained: at 6.44 ppm due to H4 hydrogen (Hazekamp et al., 2004a), and at 8.2 ppm and 6.12 ppm due the aromatic hydrogens H2 and H3' respectively. Similar results of CBN at 7.12 ppm and 7.04 ppm due to the aromatic hydrogens H5 and H4 respectively were also published (Julia et al., 2018).

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 8).

Similar results of Δ^9-THC were obtained by Hazekamp et al. (2004b) on 6.14 ppm due to H2 hydrogen; by Julia et al. (2018) on 6.12 ppm due to the aromatic hydrogen H3'; by Politi et al. (2008) on 6.14 ppm due to H3'; by Pisanti et al. (2017) on 6.15 ppm due to the aromatic hydrogen H5'.

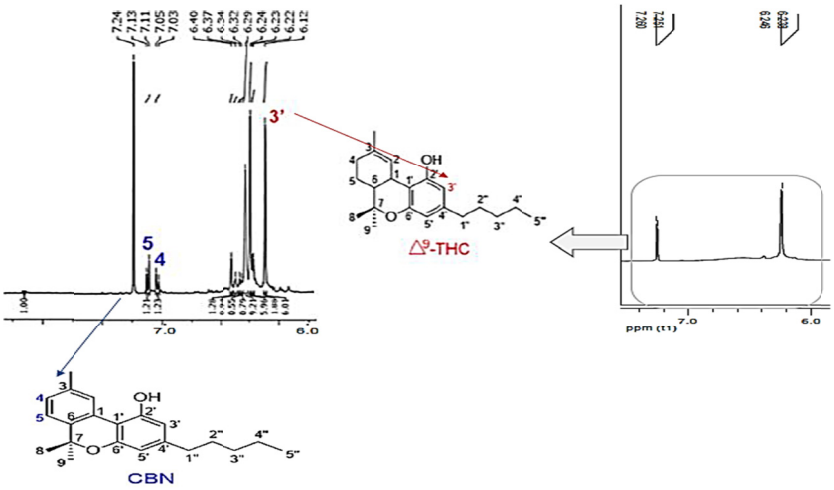

Figure 8. CBN and THC presence clarification through NMR technique

The H-9 protons give us 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 9). This reveals the place of CBD compound in the NMR spectroscopy related to the chemical structure of CBD Similar results were also obtained by Julia et al. (2018) on 4.2-4.3 ppm; by Hazekamp on 5.57 ppm due to H10 and one signal for the cis on 4.66 ppm and one for the trans configuration on 4.56 ppm due to H9 hydrogen; Also Lewis et al. (2019) obtained results on 0.8, 1.7, 1.75, 4.35, 5.5 and 6.25 ppm due to H5'', H9, H7, H10, H2 and H3' respectively; by Pisanti et al. (2017) on 0.85, 4.42 and 6.01 due to the aromatic hydrogens H5'', H10 and H3' respectively.

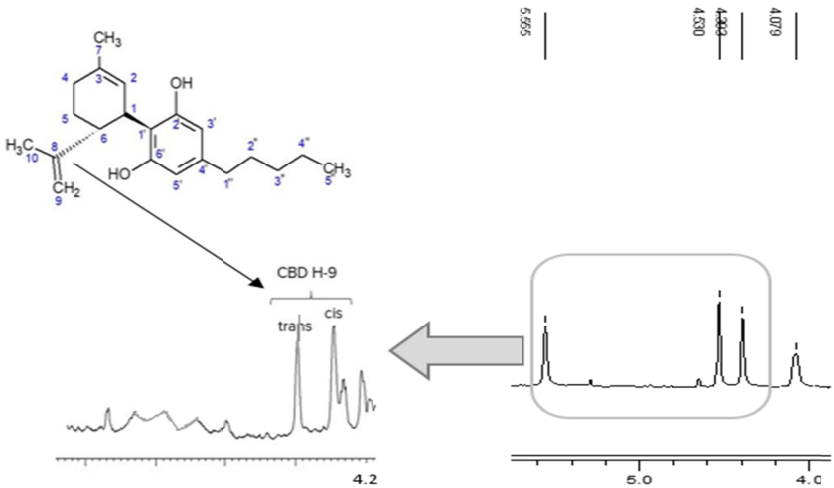

Figure 9. Double bond region showing CBD signals

According to NMR technique, the presence of the most important cannabinoids (CBG, CBD, CBN and THC) in Karma Lemon was clear. As well, it was referred that the percentage of CBDA, CBGA, CBG, CBD, CBN, 9-THC and 9-THCA in Karma Lemon cannabis strain was approved by the Food Chemistry Laboratory for The Extraction and Quantitative Analysis of the University of Naples Federico II-Department of Pharmacy, to be 92.625 mg/g, 0.184 mg/g, 0.044 mg/g, 2.077 mg/g, 0.001 mg/g, 0.545 mg/g and 0.746 mg/g respectively (Appendices). The cannabinoid profile of this strain of interest Karma Lemon was accurately determined at 0.262 indicating that Karma Lemon is an "oil and seed plant" (Chandra et al., 2017).

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.

References

Brenneisen, R. (2007). Chemistry and analysis of phytocannabinoids and other cannabis constituents. In M. A. ElSohly (Ed.), *Marijuana and the Cannabinoids*. Forensic Science and Medicine. https://doi.org/10.1007/978-1-59259-947-9

Chandra, S., Lata, H., Khan, I. A., & ElSohly, M. A. (2017). *Cannabis sativa L.: Botany and Horticulture*. Springer International Publishing AG. https://doi.org/10.1007/978-3-319-54564-6

Hazekamp, A., Choi, Y., & Verpoorte, R. (2004a). Quantitative Analysis of Cannabinoids from Cannabis sativa Using 1H-NMR. *Chemical and Pharmaceutical Bulletin, 52*, 718-21. https://doi.org/10.1248/cpb.52.718

Hazekamp, A., Ruhaak, R., Zuurman, L., van Gerven, J., & Verpoorte, R. (2006). Evaluation of a vaporizing device (Volcano) for the pulmonary administration of tetrahydrocannabinol. *J. Pharm. Sci., 95*, 1308-1317. https://doi.org/10.1002/jps.20574

Hazekamp, A., Simons, R., Peltenburg-Looman, A., Sengers, M., van Zweden, R., & Verpoorte, R. (2004b). Preparative isolation of cannabinoids from Cannabis sativa by centrifugal partition chromatography. *Journal of Liquid Chromatography and Related Technologies, 27*(15), 2421-39. https://doi.org/10.1081/JLC-200028170

Hilal, N., Bou-Karroum, L., Ataya, N., & El-Jardali, F. (2018). K_2P Rapid Response: Legalizing Cannabis Cultivation: What we need to know and is Lebanon Ready? *Knowledge to Policy (K_2P) Center*. Beirut, Lebanon.

Iseger, T. A., & Bossong, M. G. (2015). A systematic review of the antipsychotic properties of cannabidiol in humans. *Schizophrenia Research, 162*(1-3), 153-61. https://doi.org/10.1016/j.schres.2015.01.033

Jacobs, M. (2019). *The difference between cannabinoids and terpenes*. Retrieved from https://www.analytical cannabis.com

Julia, L., Marcos, O., Raphael, C., Warley, B., Rosa, R., Thalles, P. F., … Alvaro, N. (2018). Extraction and isolation of cannabinoids from marijuana seizures and characterization by 1 H NMR allied to chemometric tools. *Science and Justice, 58*. https://doi.org/10.1016/j.scijus.2018.06.005

Lewis, E. (2019). *Slow but steady progress on legalization of medicinal cannabis*. Retrieved from https://dailystar.com.lb

Pisanti, S., Malfitano, A. M., Ciaglia, E., Lamberti, A., Ranieri, R., Cuomo, G., … Bifulco, M. (2017). Cannabidiol: State of the art and new challenges for therapeutic applications. *Pharmacology and Therapeutics, 175*, 133-150. https://doi.org/10.1016/j.pharmthera.2017.02.041

Politi, M., Peschel, W., Wilson, N., Zloh, M., Prieto, J. M., & Heinrich, M. (2008). Direct NMR analysis of cannabis water extracts and tinctures and semi-quantitative data on delta9-THC and delta9-THC-acid. *Phytochemistry, 69*(2), 562-70. https://doi.org/10.1016/j.phytochem.2007.07.018

Radwan, M. M., Wanas, A. S., Chandra, S., & ElSohly, M. A. (2017). Natural Cannabinoids of Cannabis and Methods of Analysis. *Springer International Publishing, 7*, 161-182. https://doi.org/10.1007/978-3-319-54564-6_7