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HIGH-THROUGHPUT PHYTOCHEMICAL CHARACTERIZATION OF NON-CANNABINOID COMPOUNDS OF *CANNABIS* PLANT AND SEED, FROM PAKISTAN

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Abstract

The herbs are the natural resources for the infinite phenolic compounds that are used in pharmaceutical industry. These herbs are of significant importance due to their beneficial usage for the human health. Here, we studied a common herbs *Cannabis sativa*, an important member of the family Cannabaceae for phytochemical characterization. The methanol extract of whole *Cannabis* plant and seed was analyzed for the identification of non-cannabinoid compounds through High Performance Liquid Chromatography (HPLC) technique, because the non-cannabinoid compounds have not been much studied in *C. sativa.* These compounds are very useful in different diseases, used in cosmetics and as antioxidant agent. HPLC analysis revealed the presence of a variety of non-cannabinoid compounds including Quercetin, Gallic acid, p-Coumaric acid, m-Coumaric acid, Caffeic acid, Cinnamic acid, Ferulic acid, Benzoic acid and Kampferol. Furthermore, Quercetin was observed with high concentration in whole plant sample, whereas high Gallic acid and absence of m-coumaric acid was noted in the *Cannabis* seed. It was also observed that plant samples were with higher concentration of cinnamic acid as compared to seed. The Caffeic acid, Benzoic acid and Ferulic acid were in low concentration in both *Cannabis* plant and seed samples. Kampferol is another important non-cannabinoid compound that was also quantified in both samples. This research will be providing a foundation for further molecular characterization of *Cannabis* plant and seed for their beneficial usage.

Key words: *Cannabis sativa*, HPLC, Non-cannabinoids, Phytochemical analysis.

Introduction

In human history, there are many plants which are used as medicines, for synthesis of paper, clothes and for the prevention and cure of various types of ailments (Nasrullah *et al.,* 2012; Horcajada *et al.,* 2011). *Cannabis sativa* (Hemp, Local name bhang) belonging to family Cannabaceae, is an annual herbal plant that has also been used in different regions of the world as a source of oil and for other medicinal purposes (Williamson & Evans, 2000). Cannabis sativa is a potent allergy causing weed but its different parts as well as the chemical extract have been used for the leisure and therapeutic purposes for curing different diseases of mankind (Mukhtar *et al.,* 2008; Shinwari *et al.,* 2015). Initially this plant was limited to central Asia but now it is found all over the world because of its advantages and usage (Nasrullah *et al.,* 2012).

The genus *Cannabis* has three species *C. sativa*, *C. indica* and *C. ruderalis* and showed great natural distribution in the flora of Russia, China, India, Iran and in Pakistan (Hillig, 2005). *C. sativa* and *C. indica* are frequently grown all over the world while, in Pakistan they are naturally grown as wild form in mountainous and rural areas. Mostly the wild plants of *C. ruderali* and *C. sativa* showing natural distribution in the low land regions harbored low concentration of cannabinoid and non-cannabinoid compounds. However, the *C. indica* types that are commonly found in the Hindukush, North mountainous and in Karakoram regions have higher concentration of cannabinoid compounds (Anwar *et al.,* 2006). *C. sativa* is naturally grown in different parts of the Punjab province like in Sialkot, Layyah, Bhakar, Mianwali, Muzafargarh and D. G khan and in the hilly area of Islamabad, Quetta and other northern parts of Baluchistan (Aziz *et al*., 2016). As a crop, it is grown at very low scale in different areas of Pakistan and commonly known as bhang in the local language (Anwar *et al.,* 2006).

Different types of cannabinoid compounds like Cannabichromene (Claussen *et al.,* 1966), Cannabidiol (Adams & Martin, 1996), Cannabielsoin (Hartsel *et al.,* 1983) etc. have already been extracted and studied in *C. sativa.* All these identified secondary metabolites are reported to have much biological and therapeutic significance. For example, Quercetin has been used in the treatment of diabetes, cataracts, asthma, and for the prevention of cancer. Cinnamic has been used in cosmetics and in cancer intervention. Similarly, the Gallic acid has also been used in diabetes, in high blood pressure treatment, and has strong antioxidant activity. Similarly, Kampferol is highly effective in pancreatic cancer and sclerosis of various types (Abrams *et al.,* 2007).

It is also observed that, *C. sativa* from mountainous area of northern regions has a high level of cannabidiol (CBD) and tetrahydrocannabinol (THC) compounds having significant antimicrobial activity (Langezaal *et al.*, 1992). This study was carried out to perform a comprehensive phyto-chemical analysis of *C. sativa* plant sap and seeds from Pakistan so that it will be easy for further molecular characterization of this plant for the beneficial usage of it.

Materials and Methods

Plant materials: Fresh and naturally grown plants of *cannabis sativa* were collected from the area of Kacha (Layyah, Pakistan) near the bank of Indus River. Similarly, the dry seeds of *Cannabis sativa* were also purchased from a farmer of Kacha, Layyah during 2016. Table 1 and Fig. 1 representing the whole information about the samples that were used in this study.

Table 1. Sample collection.

No.	Scientific name	Local name	Plant part used
1.	*Cannabis sativa*	Bhang	Leaves, Roots, Stem
2.	*Cannabis sativa*	Bhang	Seed

Methanol extraction: Methanol was isolated separately from plants and seeds dried at room temperature. Plant materials (both dried plants and seeds) were grinded and converted into powder form by using mortar and pestle. Finally, 10 g powder sample of both plant and seed material was taken into two different sets of test tubes. Then extracted methanol was mixed with the powdered plant material of both test tubes. After fixed time, when the powdered material of both samples was properly dissolved then it was filtrated. Finally, both samples were put separately into a vacuum drying oven to dry the material and after drying 2-3 g remaining extracted material of both samples was used for further analysis.

Preparation of sample for HPLC analysis: 50g powder of plant and seed samples was dissolved in a solution containing 16ml double distilled water and 24ml methanol. After 5 minutes shaking, both samples were put into an oven for 2 hours. Finally, the material was filtered through micro filterate of 0.2-0.4 microns and sent to University of Agriculture, Faisalabad (UAF) for HPLC analysis.

High Performance Liquid Chromatography (HPLC) analysis: The HPLC analysis was performed at the Central HI Tech laboratory of the UAF, Pakistan. Gradient reverse phase HPLC (Shimadzu, Japan), was used for this study and the whole procedure was adopted according to Walsh *et al*., (2003).

Overall, the detector of SPD-10AV value was used for the analyses of bands produced by different compounds. Different components present in the mixture of samples were separated according to the procedure of Audu *et al*., (2014) by changing level of the reaction of sample with adsorbent material. Finally, the data was recorded in computer through electrical signals that were generated from chromatogram.

Results and Discussion

In fact the cannabis plant has more than 500 compounds that make it a complex matrix. Previously, the scientist have more focused on the study of plant contents and distribution of the phytocannabinoids while, the plant non-cannabinoid compounds like secondary metabolites have not been fully analyzed. For the development of more comprehensive methods to extract and trace different elements from different plant matrices great efforts have been made. Mainly two different methods, high performance liquid chromatography (HPLC) and gas-chromatography (GC) have been used in most of such researches. This study also takes the advantage of comprehensive HPLC technique to analyze and quantify the non cannabinoid compounds from *Cannabis sativa* L.

Fig. 1. (A) Plant sample and (B) seed sample of *Cannabis* from Pakistan.

Table 2. Variable concentration of different non-cannabinoids in *Cannabis* plant.

No.	Reten. time (min)	Area (mV.s)	Area (%)	Amount (g/kg)	Amount (%)	Compound name
1.	2.833	0.518	0.1	0	0.026	Quercetin
2.	4.82	16.975	1.8	0	0.68	Gallic acid
3.	17.32	108.407	11.4	0	1.44	P-Coumeric acid
4.	19.733	179.097	18.8	0	2.14	M-Coumeric acid
5.	25.127	83.973	8.8	0	2.95	Cinnamic acid

Table 3. Variable concentrations of different non-cannabinoids in *Cannabis* seed.

No.	Reten. time (min)	Area (mV.s)	Area (%)	Amount (g/kg)	Amount (%)	Compound name
1.	3.153	501.735	5.8	0	26.55	Quercetin
2.	4.747	3865.513	44.8	0	139.14	Gallic acid
3.	12.467	430.171	5	0	19.78	Caffeic acid
4.	14.447	408.63	4.7	0	43.24	Benzoic acid
5.	17.533	169.236	2	0	2.19	P-Coumeric acid
6.	21.98	161.324	1.9	0	11.59	Ferulic acid

Table 4. Concentration of Kampferol in *Cannabis* plant and seed.

No.	Reten. time (min)	Area (mV.s)	Area (%)	Amount (ng/mL)	Amount (%)	Compound name
Plant						
1.	2.933	228.743	2.6	0	70.68	Kampferol
Seed						
2.	2.867	624.678	6.9	0	193.44	Kampferol

Characterization of non-cannabinoids of the *Cannabis* plant: HPLC analysis predicted that different non-cannabinoid compounds like p-Coumaric acid, m-Coumaric acid, Quercetin and Cinnamic acid were in higher concentration in whole plant sample as compared to seed sample. The peculiar behavior of different compounds through the column of HPLC is very important to study the compound individually and in this project few peculiar behaviors like flow rate, retention time and area covered by each compound were analyzed.

It was analyzed that Quercetin had highest peak than any other compounds when detected by the chromatograph that had a flow rate about 0.026ppm with retention time 2.833 sec and area covered was 0.518 mV.s. Similarly, the flow rate of Cinnamic acid was 2.95ppm with 25.127 sec and 83.973 mV.s of retention time and area covered respectively. Meanwhile, the Gallic acid had 0.68ppm flow rate, 4.820 sec retention time and 16.975 mV.s area covered. The m-Coumaric acid and p-Coumaric acid had flow rate about 2.14ppm and 1.44ppm respectively. These results indicated that the concentration of p-Coumaric acid and m-Coumaric acid was not very high as compared to the Quercetin. The whole detail of different compounds about their flow rate, retention time and area covered in whole plant sample is given in the following Table 2.

Characterization of principal non-cannabinoids of the *Cannabis* seed: *Cannabis* seeds also contained different compounds like plant. It is observed that in seed Quercetin had flow rate about 26.55ppm with retention time and covered area of 3.153 sec and 501.735 mV.s respectively. It is observed that flow rate, Retention time and area covered by Gallic acid in seed sample was higher in concentration than Quercetin that was in 139.14ppm, 4.747 sec and 8665.513 mV.s. Similarly, the Caffeic acid and Benzoic acid had with flow rate about 19.78ppm and 43.24ppm respectively and their Retention time was 12.467 sec and 14.447 sec. with covered area of 430.171 mV.s and 408.630 mV.s. These results predicted that the concentration of Caffeic acid and Benzoic acid is lower than Quercetin and Gallic acid. Further, the level of p-Coumaric acid was also studied with flow rate 2.19ppm, time 17.533 sec and area covered 169.238 mV.s. The level and concentration of Caffeic acid was observed intermediate in nature as we identified through graphs. Different level of Ferulic acid was observed in flow rate, time and area covered that was about 11.59ppm, 21.980 sec and 161.324 mV.s. Different flow rates, Reten. time and area covered by the Gallic acid, Caffeic acid, Benzoic acid, p-Coumaric acid, Ferulic acid and Quercetin is shown in the following Table 3.

Co-comparison between non-cannabinoids of *Cannabis* plant and seed: This study un-covers the existence of non-cannabinoids and non-psychoactive compounds in both plant and seed of the *C. sativa.* It was observed that the most important compound is the Quercetin that was present in both plant and seed with different quantity. The peak of graph of Quercetin in the plant was very high comparatively which means that the Quercetin is in very high concentration in *Cannabis* plant. HPLC analysis also identified the different level of Gallic acid in the plant and seed of *C. sativa*. Overall, high concentration of Gallic acid was observed in seed sample of *Cannabis.*

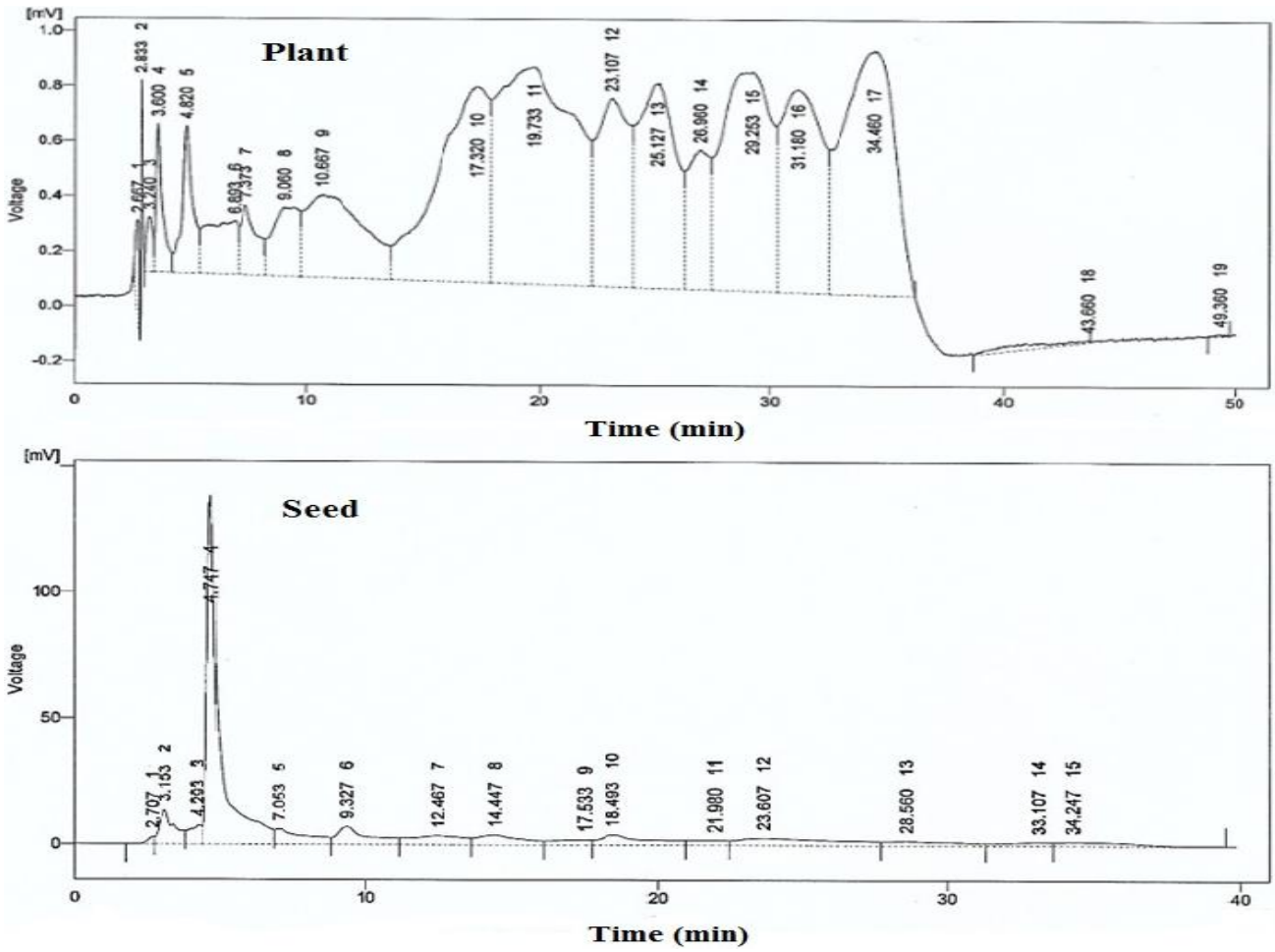

Fig. 2. Co-comparison between non-cannabinoids of *Cannabis* plant and seed.

Further, a high concentration of p-Coumaric, m-Coumaric and Cinnamic acid was quantified in plant sample than seed. Cinnamic acid and m-Coumaric acid were totally absent in seed sample. Similarly, the other phenolic compounds like Caffeic acid, Benzoic acid and Ferulic acid were in minute quantity in seed extract while all these compounds were missing in *Cannabis* plant. A brief comparison of non-cannabinoids between plant and seed extract of *Cannabis* is shown in Fig. 2.

Kampferol from the *Cannabis* plant and seed: Kampferol is an important non-cannabinoids compound that is very effective in Pancreatic Cancer and Sclerosis of various types. The quantity of this compound was also calculated separately in plant and seed sample as well. The same formula was adopted for the quantification of kampferol during HPLC analysis as we used for the remaining compounds. It is observed that the flow rate of Kampferol was 70.68ppm in plant sample with Reten. time and area covered of 2.933 sec and 228.743 respectively. Kampferol flow rate was observed as 193.44ppm with Reten. time of 2.867 sec and area covered of 624.678 mV.s in seed sample.

Overall, we could not isolate a high quantity of Kampferol in both plant and seed sap in comparison to other compounds. The whole information about the time, area covered and flow rate about Kampferol compounds in both seed and plant samples is shown in the following Table 4.

Conclusions

Conclusively, we can say that *C. sativa* plant and seed have a significant quantity of Quercetin, Gallic acid, p-Coumaric acid, m-Coumaric acid, Ferulic acid, Benzoic acid, Caffeic acid and Kampferol. So, this common herb can be used as a good source of all above mentioned non-cannabinoid compounds in medical field other than anesthetic and psychoactive purposes.

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