WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 16, 454-463. Research Article ISSN 2277–7105

PHYTOCHEMICAL, ANTIMICROBIAL AND ANTIOXIDANT PROPERTY OF *CANNABIS SATIVA*

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Article Received on
04 Oct. 2017,

Revised on 25 Oct. 2017,
Accepted on 16 Nov. 2017,

DOI: 10.20959/wjpr201716-9976

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ABSTRACT

The study is aimed to investigate the phytochemical, antimicrobial screening and antioxidant activity of different extracts of *Cannabis sativa*. Phytochemical analysis was done to estimate the concentration of tannin, protein, phenolic, carbohydrates and antioxidants on wide spectrum of compounds extracted from *Cannabis sativa*. Acetone and Methanol extracts showed relatively more antimicrobial activity than aqua extracts by exhibiting larger zone of diameter against *Agrobacterium*, *E.coli* and *Bacillus subtilis*. Methanol extracts (45.8 %) exhibited more antioxidant activity than the standard ascorbic acid (42.3 %). IC50 value of 25.63µg/ml proves that *Cannabis sativa* has high antioxidant activity even at low concentrations. TLC examination revealed the presence of CBGA- Cannabigerolic acid, THCA- (2)-D9-(trans)-tetrahydrocannabinolic acid A, CBDA- Cannabidiolic acid, CBD- Cannabidiol in acetone and methanol extracts of *C.sativa*. We performed HPLC to confirm the presence of various vital cannabinoids using specific buffer. Their time of elution confirmed the presence of Cannabidiol, Cannabinol, (-)-Δ9-THC and (-)-Δ9-THC acid. The results revealed that the plant extracts of *C.sativa* contains bioactive compounds having antimicrobial activity and so useful in human medicines.

KEYWORDS: *Cannabis sativa,* Antioxidants, cannabinoid, IC50, HPLC.

INTRODUCTION

Medicinal plants have been used from ancient time for their medicinal values as well as to impart flavour to food. Nowadays, the crude extracts and dry powder samples from medicinal and aromatic plants and their species have been shown interest for the development and

preparation of alternative traditional medicine and food additives. Plants have been the basis for medical treatments through much of human history, and such traditional medicine is still widely practiced today. Modern medicine recognizes herbalism as a form of alternative medicine, as the practice of herbalism is not strictly based on evidence gathered using the scientific method. However, to make use of many plant-derived compounds as the basis for evidence-tested pharmaceutical drugs and phototherapy works to apply modern standards of effectiveness testing to herbs and medicines that are derived from natural sources.[1]

In the 1930s and 1940s the chemical structure of the first phytocannabinoids had been successfully characterized. However, it was not until 1964 that Δ9-tetrahydrocannabinol (Δ9-THC, dronabinol), mainly responsible for the pharmacological effects of the cannabis plant, was stereo chemically defined, and synthesized. Another scientific breakthrough in cannabinoid research was the detection of a system of specific cannabinoid receptors in mammals and their endogenous ligands which constitute the cannabinoid system, within the past 15 years. About 65 cannabinoids have been detected in the *Cannabis* plant, of which cannabigerol (CBG), can-nabichromene (CBC), cannabidiol (CBD), Δ9-THC, and cannabinol (CBN) are the most relevant in quantity.[2] The THC main effects may be modulated by other cannabinoids, mainly CBD, and other cannabis constituents. In addition to these phytocannabinoids synthetic agonists and antagonists at the cannabinoid receptor and other modulators of the endogenous cannabinoid system are under investigation for potential therapeutics. In a medical context Δ9-THC is usually called dronabinol.[3]

2. MATERIAL AND METHOD

2.1 Sample collection: *Cannabis sativa* was collected from Yamuna bank (Noida, U.P). As observed this plant was found in abundance like weed. It does occupy a small area, grows in patch of land with abundance of ground water and fertility. It's not hard to identify this plant as it has a very unique taxonomy of leaf and was stored at 4˚C till use.

2.2 Sample preparation: Fresh leaves were collected from fertile soil and divided into two halves after washing it thoroughly with distilled water. First half was kept in oven for drying overnight**.** Other half was further divided into four equal halves and crushed using different solvents such as acetone W4, methanol W3, cold water W2 and hot water W1 of 10ml each. These were stored at a temperature of 4˚C in different falcon tubes. Same was done with dry sample labelled as D4, D3 D2 and D1, further concentrated by the process of filtration.

2.3 Qualitative analysis

Qualitative preliminary phytochemical analysis was performed according to.[4,5]

2.3.1 Test for Alkaloids: Wagner's test**:** 5ml of each sample were taken in clean glass test tubes to which concentrated hydrochloric acid with Wagner's reagent was added. Appearance of reddish brown precipitate confirmed the presence of alkaloids.

Tannic acid test**:** 0.5ml of 10 % tannic acid solution was added to test tube containing 2ml sample, buff (cloudy) coloured precipitation confirmed the presence of alkaloids.

2.3.2 Test for Tannins: Ferric Chloride test: 5 ml test solution was taken in a glass test tube and few drops of 10% $FeCl_3$ were added into it. After incubation blue green or black colour confirmed the presence of tannins.

2.3.3 Test for Carbohydrate: Fehling test: 2ml of Fehling solution consisting of Fehling A solution **(**1% $CuSO_4$**)** and Fehling B solution **(**25% KOH mixed with 35% Sodium Potassium Tartrate**)** was added to 2ml sample. After incubation red coloured precipitate confirmed the presence of carbohydrate.

Molish test

Few drops of alcoholic α-naphthol and few drops of concentrated H_2SO_4 were added to 5ml of each sample. After incubation a violet coloured ring confirmed the presence of carbohydrate.

2.3.4 Tests for Proteins

Nin-hydrin test: Few drops of 0.5 % nin-hydrin solution were added to the sample followed by water bath. Appearance of Purple /violet colour confirms the presence of protein.

2.3.5 Test for Saponin: Froth test: We took sample in a falcon tube and equal amount of distilled water was added into it, which was vortexed vigorously. Stable froth formation confirms the presence of saponin.

2.4. Quantitative analysis

Dried leave extract of *C.sativa* was studied for various quantitative analyses. Amount of tannin, protein, total phenolic and carbohydrate were determined in total plant extract.

2.4.1. Tannin estimation

5ml of each filtered extract was pipetted in a test tube and mixed with 2ml diluted ferric chloride in 0.1N Hydrochloric Acid and 0.008N Potassium Ferro cyanide. All the reagents in each tube were mixed well and kept undisturbed for 30 minutes. O.D. was taken at 660nm against reagent blank.[2]

2.4.2. Protein estimation: Standard solution of BSA(bovine serum albumin) was added to different test tubes making it 30, 60, 120, 240 µg respectively. Four test tubes were taken and volume was made up to 1ml using distilled water. The test tube with 1ml water serves as blank 4.5ml of reagent1 (0.1M sodium hydroxide) was added to each test tube and kept under incubation period of 1 hour. After incubation 0.5ml of reagent2 (4% cupric sulfate) was added and incubated in a dark room for 30 minutes. Thereafter absorbance was taken at 660nm and standard graph was plotted. The amount of protein present in the given sample was estimated from the standard graph.[4]

2.4.3. Carbohydrate estimation: Anthrone reagent was prepared by dissolving 0.2g of anthrone in 100ml sulphuric acid. 4ml of anthrone reagent was added and mixed along with other contents. Test tubes were kept in water bath for 10 min. O.D was measured at 620nm.[6]

2.4.4. Total Phenolic Estimation: FC (Folin Ciocalteu) reagent was added to an aliquot (2ml) of the extract with Sodium Carbonate solution. The samples were thereafter incubated in a thermostat at 45^{o}C for 45 min. The absorbance was determined using spectrophotometer at 660 nm.[7]

2.5. Antimicrobial activity: Anti-microbial activity was studied using well diffusion techniques on nutrient agar plates. Pathogens used for testing antimicrobial activity were *E.coli*, *Agrobacterium* and *Bacillus Subtilis* which were prepared by using 24 hours old cultures. Wells were bored with sterile cork borer and were filled with 100 µl of each of 8 extracts. Extracts showing maximum anti-microbial activity were further analysed through TLC for compound identification.

2.6. Thin layer chromatography: 10µl of each sample W3, W4, D3 and D4 were loaded on each plate and kept for drying. Running solution was prepared by predicting the types of component polar or non-polar. After running mobile phase the plates were allowed to dry in a stable condition. Bands were observed using iodine crystals. A solution of Chloroform, Ethyl

acetate and Formic acid in the ratio of 10:9:2 respectively were used as mobile phase for separation of polar compounds. Similarly Benzene, Methanol and Ammonia in the ratio of 18:2:0.2 was used for elution of non-polar compounds.[8,9]

2.7. Antioxidant Test

The stock solution of dried plant extract was prepared in methanol to achieve the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99 and 0.97 µg/ml. Diluted solutions (1 ml each) were mixed with 1 ml of methanol solution of DPPH (2, 2-diphenyl-1-picryl hydrazyl). After 30 min incubation in darkness at room temperature (23^{o}C), the absorbance was recorded at 517 nm.[10,11] Ascorbic acid was used as standard. IC50 value was estimated by calculating the percent scavenging activity of free radicals by absorbance. DPPH radial scavenging activity in this experiment was calculated as follows

Scavenging activity % = [{absorbance (control)– absorbance (sample)} / absorbance (control)]*100%.

2.8. High Performance Liquid Chromatography (HPLC)

The sample was extracted using methanol/chloroform and introduced in a discrete small volume into the stream of mobile phase percolating through the column. The time at which a specific compound elutes (emerges from the column) is called its retention time. The retention time measured under particular conditions is considered an identifying characteristic of a given analyses.[12]

3 RESULTS

3.1 Phytochemical analysis (qualitative)

Qualitative analysis of various phytochemical parameters is given in the Table1. From a single test we cannot conclude the nature of secondary metabolites. So, for more experimental evidence more than one color test was conducted in most of the cases.

Table. 1: Phytochemical analysis of *Cannabis sativa* leaf extracts in different solvents. Where, D-Dried extract and W-Fresh extract dissolved in D1, W1-Hot water; D2, W2-Cold water; D3, W3-Methanol; D4, W4-Acetone.

Tests	D1	D2	D3	D4	W1	W2	W3	W4
Alkaloid test								
Wagner's	+	+	+	+	+	+	+	+
Tannic acid test	+	+	+	+	+	+	+	+
Tannins								
Faric-chloride	+	+	+	+	+	+	+	+
Carbohydrate test								
Fehling	-	-	-	-	-	-	-	-
Molisch test	+	+	+	+	+	+	+	+
Protein								
Ninhydrin	+	+	+	+	+	+	+	+
Saponin								
Froth test	+	+	+	+	+	+	+	+

3.2 Quantitative analysis

After calculations average concentration of tannin, protein, carbohydrate and phenolic present in dried leaf extract of *C. sativa* is shown in Table 2.

Table. 2: Quantitative analysis of various phytochemical parameter.

S. No.	Compound	Concentration (µg/ml)
1	Tannin	56.82
2	Protein	22.56
3	Carbohydrate	401.42
4	Phenolic	16.57

3.3Antimicrobial activity: On studying anti-microbial activity and noting the diameter of effected area we can plot the following graph. Acetone and Methanol extracts showed relatively larger zone of diameter than aqua extracts. Acetone and Methanol extracts exhibited large zone of diameter against *E.coli, Agrobacterium and Bacillus subtilis* than the positive control.

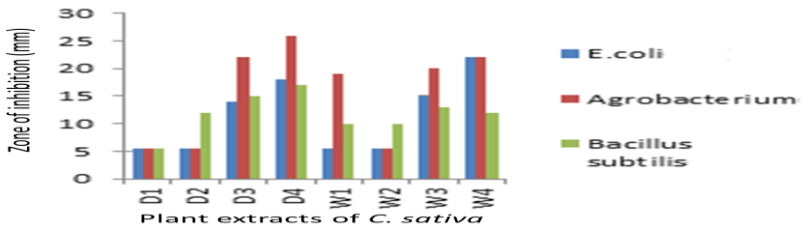

Figure1: Antimicrobial activity of *Cannabis sativa* extracts in different solvents against *E.coli, Agrobacterium and Bacillus subtilis.*

3.4 Thin layer chromatography

Relative retention (Rf) values of the cannabinoids in a polar (silica-gel) and non-polar TLC-system. The colour of chromatographic spots after spraying indicates the presence of various cannabinoids. According to the retention factor various cannabinoids were primarily identified as polar and non- polar cannabanoids given in the Table 3.

Table 3: Identification of various polar and non-polar cannabinoids on the basis of their retention relative (Rf).

Sample	Retention Time (Rf)	Cannabinoid
W3	0.67	CBGA
W3	0.40	THCA
W3	0.32	CBGA
W4	0.69	CBDA
W4	0.55	CBD
D4	0.69	CBDA
D3	0.67	CBGA

CBGA- Cannabigerolic acid, THCA- (2)-D9-(trans)-tetrahydrocannabinolic acid A, CBDA-Cannabidiolic acid, CBD- Cannabidiol.

3.5 Antioxidant activity

DPPH radial scavenging activity of standard ascorbic acid was 42.3% and scavenging activity of methanol extract as 45.8%. Using OD value attained from spectral analysis we can easily find out %inhibition therefore IC50 value of the sample. From the graph between concentration and percent inhibition IC50 value of our sample is found to be 25.63µg/ml.

3.6 HPLC

The results of HPLC quantification cannabinoids in the extracted plant extract. Analysis of HPLC pictogram according to the guide lines of United Nations Office on Drugs and Crime (UNODC) various cannabinoids were identified by retention time. Area under the peak provides quantitative data regarding those cannabinoids. Figure 2 shows peaks for various compounds present in our sample and area under the curve provided quantitative information about various cannabinoids as mentioned in Table 4.

Table. 4: The eluted sample was distinguished on the bases of their Rt (retention time) and the area under the curve provide the quantitative data of various cannabinoids.

S. No.	Name of cannabinoids	Retention Relative (min)	Area units
1	Cannabidiol	4.821	838025
2	Cannabinol	5.483	161256
3	(-)-Δ9-THC	6.767	21165
4	(-)-Δ9-THC acid	7.730	105227

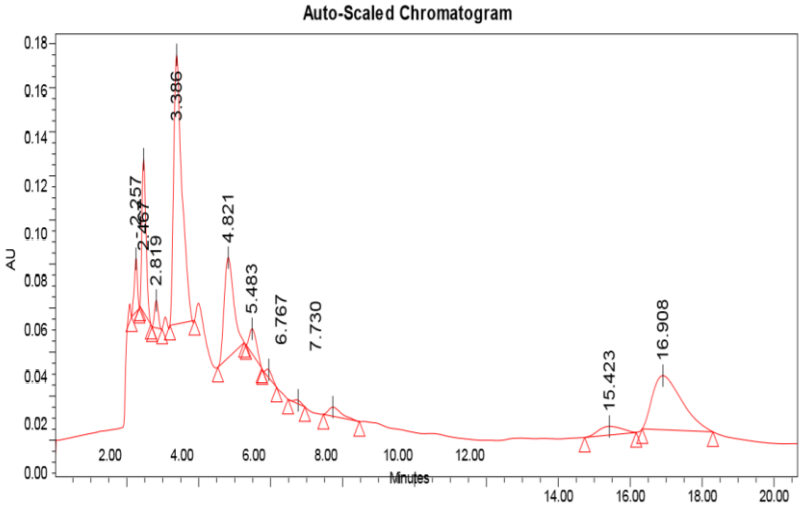

Figure. 2: Identification of cannabis extract by HPLC Chromatogram.

DISCUSSION

Qualitative analysis provides us a wide spectrum of compounds present in our sample. Various methods were followed for estimating the concentration of tannin, protein, phenolic, carbohydrates and antioxidants. Results of High performance liquid chromatography (HPLC) confirmed the presence of Cannabidiol, Cannabinol, (-)-Δ9-THC and (-)-Δ9-THC acid eluted at specific pressure and using specific buffer. This gives us an approximate concentration of the essential compounds present which are biologically active and have been used in traditional medicines for thousands of years.[13,14,15] Cannabinoids has been used in the treatments of chemotherapy-associated nausea and vomiting. They are also used to stimulate the appetite and increase food intake in AIDS and cancer patients. They have been tested in the treatment of cancer cells and are found to significantly inhibit cancer cell growth. Antimicrobial activity is performed on plates using well diffusion technique helping us to study cytotoxic chemical properties. Antimicrobials of plant origin have enormous therapeutic potential and these plants may offer a new source of antibacterial, antifungal and antiviral agents with significant activity against infective microorganisms.[15,16,17] Methanol extracts of *Cannabis sativa* were screened for antioxidant activity and DPPH radical

scavenging activity was found to be 45.8%. This study suggests that these plants possess antioxidant activities which can counteract the oxidative damage induced by free radicals.

CONCLUSION

It may be concluded that the plant *C.sativa* is enriched with significant antimicrobial and antioxidant activity due to the presence of active constituents like phenolic compounds, tannins and cannabinoids, thereby justifying their use in the indigenous system of medicine.

ACKNOWLEDGEMENT

The authors are grateful of Director of UIET, Kurukshetra University, Kurukshetra, India, for providing instruments and facilities for this research.

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