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PHYTOCHEMICAL AND ANTIOXIDANT ACTIVITY OF *CANNABIS SATIVA* L.

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ABSTRACT

There are different chemicals in plants as well as also in *Cannabis sativa* L. The aim is to identify the phytochemical and anti-oxidants that are present in *Cannabis*. For this study different tests were performed with ethanolic extract of leaves of *Cannabis sativa*, it is proven that there are number of chemicals, that are present in *Cannabis*. The highest value of absorbance of total phenolic content is 0.61±0.06 µg/ml, highest absorbance value of total flavonoid content is 0.65±0.07 µg/ml and %age inhibition of DPPH radical scavenging activity of leaf extract is 58.1 µg/ml. Our study suggested that leaf of *Cannabis sativa* are a good source of natural antioxidant.

INTRODUCTION

The study of phytochemicals derived from plants is known as phytochemistry. The plant *Cannabis* has a large amount of chemicals about 560 compounds are known and 120 compounds or cannabinoids are identified. Female plants produce more compounds as compared to male plant. The plant contains psychoactive chemicals known as cannabinoids, the most abundant of which is 9-THC, which is generated predominantly in the leaves and flower buds of plant. Aside from 9-THC, there is a number of cannabinoids with therapeutic activities that are non-psychoactive, such as cannabigerol (CBG) cannabidiol (CBD), and cannabichromene (CBC) as well as other non-cannabinoid components from several natural product groups (ElSohly, 2017).

The most significant chemical component is delta-9-tetrahydrocannabinol, which interacts with other cannabinoids and cannabinols to produce synergistic effects. Chemical composition of *cannabis* is complex because it contain more than five hundred chemicals, (sugars, hydrocarbons, mono and sesquiterpenes, steroids, flavonoids, amino acids and nitrogenated complexes. The main cannabinoids are δ-8-tetrahydrocannabinol (δ-8-THC, $C21H30O2$, 314.46 g/mol), cannabinol (CBN, $C21H26O2$, 310.43 g/mol), cannabinodiol (CBND, $C21H26O2$, 310.43 g/mol), δ-9-tetrahydrocannabinol (δ-9-THC, $C21H30O2$, 314.45 g/mol), cannabiciclol (CBL, $C21H30O2$, 314.46 g/mol), cannabidiol (CBD, $C21H30O2$, 314.46 g/mol), cannabicitran (CBN, $C21H30O2$, 314.5 g/mol), cannabichromene (CBC, $C21H30O2$, 314.46 g/mol), cannabigerolmonomethyl ether (CBGM, $C23H34O4$, 374.5 g/mol), cannabigerol (CBG, $C21H32O2$, 316.48 g/mol), dehydrocannabifuran, (DHC, $C21H24O2$, 308.41 g/mol), cannabielsoin (CBE, $C21H30O3$, 330.46 g/mol), and cannabitriol (CBT, $C21H30O4$, 346.46 g/mol) (Barrale-cureno *et al.,* 2020).

The δ-9-THC is the cannabinoid with the maximum psychotropic potency, and it is soluble in lipids because to its hydrophobic characteristics. δ-8-THC has a similar pharmacological profile as δ-9-THC, while the effects of δ-8-THC are significantly less and found at low concentrations in most types (Zuardi *et al.,* 2006).

Terpinolene, α-pinene, β-myrcene, trans-ocimene and sesquiterpene-transcaryophyllene are the most common monoterpenes. The important oils that have the major terpens of five distinct European cultivars of *Cannabis* include, trans-caryophyllene (12.2-18.9 percent), pinene (7.2-14.6 percent), humulen (6.1-8.7 percent), terpinolene (7.0-16.6 percent) and myrcene (21.1-35.0 percent) (Novak *et al.,* 2001).

Other terpenoids found in trace quantities include trans-β-farnesene pulegone, sabinene, viridiflorene, 1,8-cineole (eucalyptol), alloaromadendrene, γ-terpinene, bornyl acetate, γ-cadinene, α-copaene, trans-nerolidol, terpineol-4-ol, terpinene, β-bisabolene (McPartland and Russo, 2001).

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MATERIALS

Equipments

Grinder, Brushes, Plant spreading panels, Jars or containers, Stirrer,Conical flasks (250ml, 500ml, 1000ml), Measuring cylinder (1000ml, 500ml, 100ml), Tripod stand, Whatman filter paper no. 1, 2, Funnel, Test tubes, Test tube holder, Spirit lamp

Chemicals

Ethanol (chemical), HCL, Mayer's reagent, Water, Ferric chloride ($FeCl_3$), Concentrated Ammonia (NH_3), Benzene, Chloroform, Concentrated Sulfuric acid (H_2SO_4), Diluted Sodium Hydroxide (NaOH), Million's reagent, Gallic acid, Folin-Ciocalteu reagent (FC), Ascorbic acid,Sodium Carbonate (Na_2CO_3), AluminiumTrichloride($AlCl_3$), Sodium Hydroxide (NaOH), Sodium Nitrate ($NaNO_2$), BHT Solution, DPPH.

METHODOLOGY

Preparation of Extract

Extracts were prepared by following methods as; 250-gram powder of leaves of *Cannabis* was dipped in 700ml ethanol for seven days. After 7 days the material that was dipped was filtered by Whatmann filter paper to obtain appropriate filtrate. Filtration method was repeated three times to totally finish the plant material. This ethanolic extract was dried up by using rotator evaporator at a temperature lower than 40 ^{0}C. The final residue collected was a thick paste and saved at 4 degrees centigrade. (Ahmed *et al.,* 2019).

Phytochemical Screening (Qualitative analysis)

Test for Alkaloids

Add 2ml of 2% HCL in 0.5 ml of leaf ethanolic extract in a test tube for 15 min by using a water bath at 100 ^{0}C. After cooling add a few drips of Mayers reagent in the mixture. The appearance of yellow precipitate indicates that alkaloids are present (Siddiqui and Ali, 1997).

Test for Glycosides

2ml ethanolic extract of *cannabis* was taken and included 2ml of distilled water and 2ml of 5% ferric chloride ($FeCl_3$). Then 1 ml benzene was added to the mixture after it was heated in a water bath, and it was allowed to settle for 1 minute after shaking. 4–5 drops of concentrated ammonia (NH3) were then added. The presence of glycosides is indicated by a pink or red colour (Siddiqui and Ali, 1997; Siddiqui*et al.,* 2009; Sofowora, 1993).

Test for Terpenoids and Steroids

2 ml of chloroform was added in 1ml of ethanolic leaf extract and then a few drops of concentrated sulfuric acid (H_2SO_4) were applied to the insides of the test tubes with care. The presence of terpenoids is indicated by the appearance of a pink or pinkish brown colour (yellow), while the presence of steroids is indicated by the appearance of a blue or bluish green colour, and the presence of both terpenoids and steroids is confirmed by the appearance of both pink and blue/bluish green colours (Siddiqui and Ali, 1997).

Test for Flavonoids and Flavones

The 3 ml ethanolic extract solution received 2 ml of diluted sodium hydroxide (NaOH), which coloured the solution yellow. Then 1 mL of 5N hydrochloric acid (HCl) was added to solution which converted it colourless, indicating flavonoids, and an orange colour, indicating flavones (Siddiqui and Ali, 1997; Siddiqui *et al.,* 2009; Sofowora, 1993).

Test for Tannins

One mL distilled water and 1-2 drops ferric chloride (FeCl3) was added to 0.5 mL ethanolic plant extract to estimate tannins. Gallic and catecholic tannins are indicated by the appearance of a blue and green/black colour, accordingly (Iyengar, 1995).

Test for Proteins

A test tube was filled with 2ml of ethanolic extract then added 1ml of Million’s reagent, emergence of red colour indicate the existence of proteins.

Test for Phenols

One ml ethanolic extract solution was mixed with 2 mL distilled water and a few drops of 10% ferric chloride to detect phenols (FeCl3). The presence of phenols is indicated by the appearance of a green, blue, or white colour (Trease *et al.,* 2003).

Detection of Antioxidant Activity

Antioxidant investigation of *Cannabis sativa* was completed in the laboratory of Mirpur University of Science and Technology, Botany Department, Bhimber (AJK). For determination of antioxidant activities subsequent procedures were used: Total phenolic content (TPC), Total flavonoid content (TFC), DPPH radical scavenging activity

Preparation of Stock Solution

For analysis of antioxidant assay stock solutions of dried leaves of *Cannabis sativa* were prepared. For 1000µg /mg of stock solution, 0.2g of extract of plant was dissolved into 20ml of ethanol. From this stock solution various concentrations such as 1000µg/mg, 500µg/mg, 250µg/mg, 120µg/mg, and 50µg/mg were prepared. (Table 3.1)

Table 1: Preparation of different dilutions.

Conc. Required (µg/ml)	Amount of stock solution used (µl)	Quantity of ethanol used (µl)	Final volume of dilution (µl)
1000	1000	0	1000
500	500	500	1000
200	200	800	1000
120	120	880	1000
50	50	950	1000

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Determination of Total Phenolic Content (TPC)

Preparation of 10% sodium carbonate

10 gram of Sodium Carbonate was correctly weight and mixed in distilled water completely rising the volume up to 100 ml.

Folin-Ciocalteu (FC) reagent preparation

FC is a reagent of brilliant yellow colour. It is made out of hydrochloric acid (HCL), lithium sulfate ($Li_2SO_4H_2O$), phosphoric corrosive (H_3PO_4), bromine, sodium tungstate ($Na_2WO_4.2H_2O$), sodium molybdate (Na_2MoO_4) and water.

Standard curve of gallic acid preparation

The serial dilution approach was used to generate various fixations (50, 120, 200, 500, and 1000 g/mg) of standard solutions such as gallic acid. To increase the final volume by 1000L and at varied concentrations, precisely weigh the amount of solute with the addition of ethanol. The absorbance of sample was measured by using UV-spectrophotometer at constant wavelength 715 nm.

Procedure

With minor changes, the total phenols present in the crude extract of C. sativa were determined using the Folin-Ciocalteu reagent method. About 100µg/ml of plant extracts were mixed with 2.8 ml of 10% sodium carbonate (Na_2CO_3) and 0.1 ml of Folin-Ciocalteu reagent and were then added and incubate for 40 minutes at room temperature, at 725 nm the absorbance was measured.

Determination of Total Flavonoid Content (TFC)

Preparation of 5% sodium nitrate ($NANO_2$) solution

The correctly weighted 5g of Sodium nitrate was dissolved in to small quantity of distilled water by constantly shaking and increasing the capacity to 100 ml by adding more distilled water.

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10 % Aluminum chloride ($AICI_3$)

Preparation Take a small quantity of distilled water in beakers add 10g of $AICl_3$ was accurately weighted and stirrer and by adding more water making the up to 100 ml.

Preparation of 1molar sodium hydroxide

Four (4 g) of NaOH was weighed and dissolved in a little amount of distilled water to make a volume of 100 ml.

Standard curve preparation

Different concentrations (50,120,200,500 and 1000µg/mg) of standard solution such as ascorbic acid was prepared by using serial dilution method. The accurately weighted solutes were mixed by adding ethanol to raise the volume up to1000 µl. The standard curve was obtained on the Microsoft World 2007 by plotting absorption concentration graph of result and the sample absorbance was measured by via UV- spectrophotometer at 510 nm.

Procedure

The total Flavonoid Contents of *Cannabis sativa* was find out by Tollowing technique of Dewanto et al. (2002). According to this method taking 250 µl of plant dilution into test tubes from 500 µl of prepared plant dilution, 5% Sodium nitrate were mixed by adding 250 µl distilled water and then incubate this mixture at room temperature for 5 minutes. After 5 minutes adding 5µl 0f 10% $AlCl_3$ 500µl of 1M NaOH in a test tube and again incubate at a room temperature for 6 minutes. Afterwards there was more addition of 275 µl of refined water to raise the final volume up to 2.5 ml. The sample's absorbance was measured at 510 nm.

Determination of DPPH Radical Scavenging Activity

Preparation of DPPH solution of about 0.1mm.

As 0.00394g of DPPH transferred into a beaker and raises the volume up to 100ml with ethanol. This can be stored at $4^{o}C$.

Preparation of BHT solution

Different concentrations (50, 120, 200,500 and 1000 µg/ml) of standard solution such as BHT was prepared by using serial dilution method, the accurately weighted solutes were mixed by adding methanol to raise final volume up to 1000 µl.

Principle

DPPH is nitrogen based stable organic radical containing lambda maximum of 515nm. It is basically purple color but when sample is mixed with DPPH its purple colour become lighter and it reveal maximum antioxidant potential.

Procedure

Accurately measured plant dilution of about 1000µl was transferred in a test tube and freshly prepared DPPH of about 2.5ml was added in it. All of the test tubes were well shaken before being left at room temperature for 45-60 minutes. After an hour, the reaction between the DPPH and solution occurs, and the colour of the solution changes from purple to yellow. Spectrophotometer can be used for calculation of same results at 515nm.

Result analysis

The % DPPH value was found out by given formula.

$$\%\text{age inhibition} = \frac{\text{Control A} - \text{Sample absorbance}}{\text{Control A}} \times 100$$

Control A (Negative control) = 0.596

RESULTS

Extract of *Cannabis*

Ethanol was used to make extract of *Cannabis sativa* L. The extract was sticky and slowly flows and it shows shiny black colour.

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Phytochemical Analysis

Phytochemical analysis of *Cannabis sativa* L. show the presence of different constituents, that are given in table below (Table 2)

Table 2: Showing presence and absence of different chemicals in *Cannabis sativa.*

Constituents	Observations	Presence
Alkaloids	Yellow precipitate	+
Glycosides	Pink or red colour	-
Trepenoids	Pink or pinkish brown colour	-
Steroids	Blue or bluish green colour	+
Flavonoids	Colourless	++
Flavones	Orange colour	-
Tannins	Blue and green black colour	+
Proteins	Red colour	++
Phenols	Green or blue colour	+

++ shows high presence, + shows moderate presence, - shows absence

ANTIOXIDANT ACTIVITY

Total Phenolic Content (TPC)

The total phenolic contents of various extracts of *C. sativa* leaves varied a little. The highest and lowest phenolic content of C. sativa leaves were measured and recorded in the table given below (Table 3). Graphical representation is shown in Fig. 4.1.

Table 3: Showing TPC in different dilutions of leaf extract of *Cannabis sativa* L.

		Absorbance at different concentrations(µg/ml)				
Plant part	Fraction	50	120	200	500	1000

Leaves	Ethanol	0.23±0.03	0.32±0.09	0.38±0.06	0.46±0.06	0.61±0.06
Gallic acid	Ethanol	0.27±0.04	0.36±0.04	0.42±0.05	0.61±0.05	0.74±0.06

Results are presented as the mean values ± standard deviation.

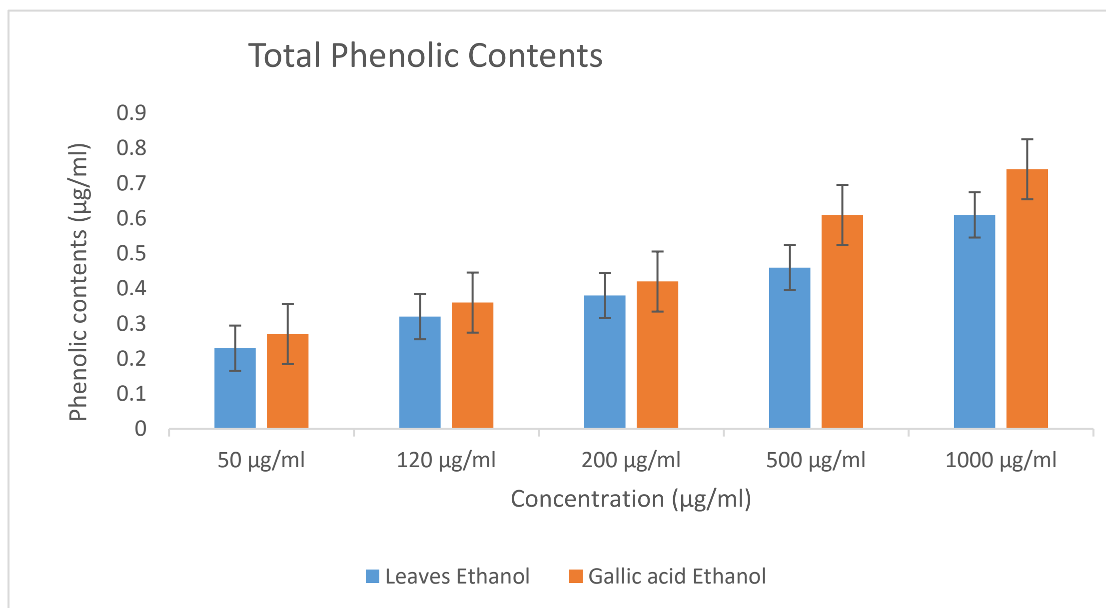

Fig. 1: Graphical demonstration of TFC in leaf extract of *cannabis sativa* L.

Flavonoid Content (TFC)

The TFC (total flavonoid content) in ethanol leaves extracts of *C. sativa* were measured and values are given in the table below (Table 4.4). also represented as graphically in Fig. 4.2.

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Table 4: Showing TFC in different dilutions of leaf extract of *Cannabis sativa* L.

		Absorbance at different concentrations (µg/ml)				
Plant part	Fraction	50	120	200	500	1000
Leaves	Ethanol	0.25±0.06	0.35±0.07	0.45±0.08	0.56±0.07	0.65±0.07
Ascorbic acid	Ethanol	0.28±0.07	0.37±0.05	0.46±0.06	0.58±0.05	0.67±0.05

Results are presented as the mean values ± standard deviation.

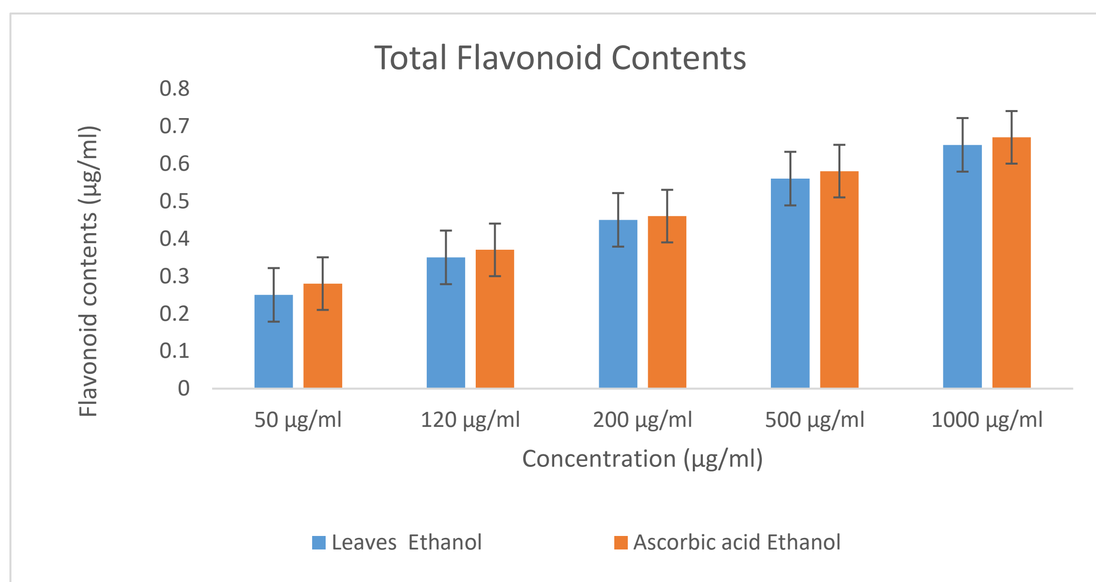

Fig. 2: Graphical representation of TFC in the leaf extract of C*annabis sativa* L.

DPPH Radical Scavenging Activity

Using a spectrophotometer, DDPH, a stable and free radical that is easily dissolved in methanol, showed distinctive colour absorption at 515 nm. Due to the donation of hydrogen molecules by antioxidant molecules, free radicals are scavenged, and the colour of the DPPH assay solution changes to a bright yellow colour, resulting in a drop in absorbance. The DPPH radicals are commonly used to measure free radical scavenging activity. Data on the 1,1-Diphenyl-1-

picrylhydrazyl (DPPH) scavenging activity of free radical of leaves of *C. sativa* are represented in (Table 4.4). Graphical demonstration is also given in Fig. 4.3.

Table 5: Showing %age inhibition in leaf extract of *Cannabis sativa* L.

		%age inhibition (µg/ml)				
Plant part	Fraction	50	120	200	500	1000
Leaves	Ethanol	11.1	22.8	31.2	41.3	58.1
BHT	Ethanol	14.4	26.1	34.5	47.9	63.1

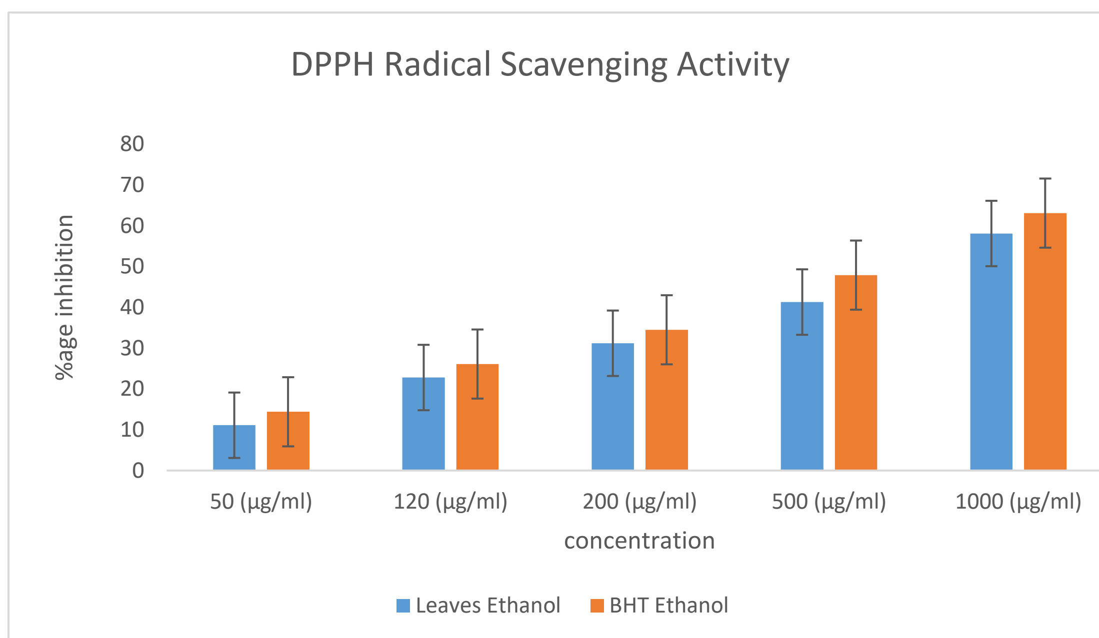

Fig. 3: Graphical representation of %age inhibition of DPPH radical scavenging activity of leaf extract of *Cannabis sativa* L.

DISCUSSION

The method of extraction, the solvent utilized, and the chemical characteristics of the molecules all play a role in secondary metabolite extraction. The analytical observation of leaf extract of *C. sativa* shows the presence of Glycosides, alkaloids, terpenoids, flavones, flavonoids, steroids, phenols, proteins and the absence of terpenoids. Audu *et al*., (2014) published the relevant

findings, reporting the existence of phytochemicals in the leaves and roots of *C. sativa* except saponins and phenols.

These isolated components like Flavonoids are revealed as significant antioxidants (Kumar et al., 2008). Phenols are biologically active chemicals that operate as powerful antioxidants and scavengers of free radicals. The maximum phenol and flavonoid content measured by methanol from C. sativa was 36.42 and 59.03 mgg-1, respectively, which agrees with Abd-Alla and Haggag (2013), who found total phenol and flavonoid contents of 9.62 mgg-1 and 1.9 mgg-1, respectively, in the leaf extract of C. sativa, with 14.5 percent antioxidant activity. The concentration of phenols, flavonoids, and antioxidant capabilities differ in different regions of C. sativa, according to the findings of several studies. Plants are a rich source of useful chemicals and other bioactive components with pesticidal properties against a wide range of insects. (Koul and Walia, 2009).

Twenty medicinal plants were tested for phenol, flavonoid, and antioxidant activity by Salama and Al Rabiah (2015). Results of phytochemical analysis of *C. sativa* showed the presence of various phytochemicals along with flavonoid contents, antioxidant activity and phenols. However, there is a scarcity of data from prior studies on extraction and comparing extract yields using different solvents, utilization of bioactive components as a substitute for manufactured chemicals from these plants, quantitative and qualitative analysis and antioxidant activity. Synthetic agents also release a lot of residues in the environment and in naturally grown populations, which is a big problem in today's agro-ecosystem. As a result, the study was carried out to examine extract yields from crude extracts of study plants using five different solvents by solvent extraction method, to observe physical properties of extract, to scrutinise bioactive compounds and to quantify total phenol, flavonoids contents, and to assess antioxidant activities by DPPH radical scavenging from crude extracts of study plants.

CONCLUSION

After conducting the present work, it seems that *Cannabis sativa* L. has very effective chemicals, like sterols, alkaloids, tannins proteins, phenols and flavonoids. Hence the studied plant is a rich source of phytochemicals, deliberating its interesting antioxidant activity. These chemicals can also reduce the blood glucose level and body weight and also used for different diseases. Its

continuous use can repair the β- cells of pancreas. There are many side effects of allopathic medicines so it is highly recommended that one should use the herbs instead of using allopathic medicines.

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