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In vitro Total Antioxidant and Radical Scavenging Activities of Organic Extracts from Leaves, Stem and Inflorescence of *Cannabis sativa* L.

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The *in vitro* total antioxidant and radical scavenging properties of organic extracts from leaves, stem and inflorescence of male and female plants of *Cannabis sativa* were studied using 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging, total phenolic conents (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, lipid peroxidation inhibition and metal chelating activity assays. The stem, leaves and inflorescence of male (MS, ML and MI, respectively) and female (FS, FL and FI, respectively) plants were initially extracted in methanol and subsequently partitioned in *n*-hexane, chloroform, ethyl acetate and 1-butanol, successively. Employing ABTS radical scavenging activity assay the fractions obtained in polar solvents exhibited high ABTS scavenging activity. Trolox equivalent antioxidant capacity (TEAC) values obtained for various extracts of different parts of *C. sativa* ranged from 144.46-1.47 mM trolox equivalents for 1-butanol fraction of FI and chloroform fraction of FS, respectively. Total phenolic contents using Folin-Ciocalteu's method ranged from 3.562-0.339 mg/L gallic acid equivalent for 1-butanol fraction of FS and chloroform fraction of MI, respectively. A direct relationship between Trolox equivalent antioxidant capacity and total phenolic contents values was not observed for the extracts except for MS indicating that only phenolic compounds were not responsible for the total antioxidant activity of the fractions. The rate of scavenging of DPPH radical for these extracts reflected the presence of a diverse nature of antioxidative components. Using ammonium thiocyanate method, all the extracts of both the genders demonstrated significant lipid peroxidation inhibition activity. The per cent chelating activity using ferrozine as reference chelator ranged from 9.46-84.94 for ethyl acetate fraction of ML and methanol fraction of FL, respectively. A poor correlation of ferrous ion chelating activity with total phenolic conents of the extracts was observed and this indicates that phenolic compounds might not be the main chelators of iron ions.

Key Words: *Cannabis sativa*, Total phenolic conents, Trolox equivalent antioxidant capacity, Lipid peroxidation inhibition.

INTRODUCTION

Historically *Cannabis sativa* L. (family: Cannabaceae) has been a very important plant for the mankind. It has been claimed to be the first cultivated plant that is used for paper, fabric, food and cure to many diseases[1]. It is a dioecious annual with distinctive aroma. It is commonly found as a weed at the peripheries of cultivated fields. Typically, the male plant is somewhat taller and more obviously flowered. The female plant exhibits a more robust appearance due to its shorter branches and dense growth of leaves and flower-associated bracts[2].

A number of secondary metabolic compounds have been identified in the genus Cannabis. Terpenes are found in abundance in *C. sativa*[3,4] and are accredited for the characteristic odor of the plant[5]. Various alkanes[6-9], nitrogenous compounds[10,11], flavonoids[12-14] and other diverse compounds[15,16] have been identified. Cannabinoids, the phenolic compounds that predominantly exist as carboxylic acids[17-20], are considered to be unique to this genus. A large number of cannabinoid and non-cannabinoid constituents have been identified in the plant[21-23]. Some isolates displayed weak to strong antileishmanial[22,23], antimalarial, antimicrobial and antioxidant activities[22]. Delta(9)-tetrahydrocannabinol (delta(9)-THC), the principal psychoactive constituent of the *C. sativa* plant and other agonists at the central cannabinoid [CB(1)] receptor has been suggested to induce psychomotor effects, psychotic reactions and cognitive impairment resembling schizophrenia[24]. The safety and antiemetic efficacy of delta-9-tetrahydrocannabinol in patients receiving cancer chemotherapy have been discussed and critically evaluated[25].

Effects of cannabidiol, a non-psychoactive constituent of *C. sativa* with antiinflammatory properties was demonstrated to restore liver function, normalize 5-HT levels and improve brain pathology in accordance with normalization of brain function in the liver and brain[26]. Neuropathy is the most common complication of diabetes and it is still considered to be relatively refractory to most of the analgesics. The beneficial effects of cannabis extract have been demonstrated in attenuating diabetic neuropathic pain, possibly through a strong antioxidant activity and a specific action upon nerve growth factor[27].

In spite of great deal of research on the medicinal and other uses of the chemical constituents of *C. sativa*, the literature is mute about any comprehensive study regarding *in vitro* total antioxidative capacity of the plant. The present research is focused on evaluating the total antioxidative activity of extracts from *C. sativa* using existing analytical assays.

EXPERIMENTAL

All the reagents and solvents used were of the highest purity grade available. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, linoleic acid, Folin-Ciocalteu's reagent (FC reagent) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Fluka (Switzerland) and HPLC-grade ethanol from Rathburn Chemicals Ltd. (Walkerburn, Peebleshire, Scotland). Potassium persulfate (di-potassium peroxdisulfate), iron(III) chloride, ascorbic acid and gallic acid were obtained from Merck Co. (Darmstadt, Germany). Double-distilled water was used throughout the experimentation. All the experiments were conducted on UV-visible spectrophotometer, UVD-3200, Labomad, Inc., USA, equipped with temperature control system. The experiments were performed thrice and all the measurements were made in triplicate. The data obtained were averaged and incorporated in the bar or line graphs for comparison purposes.

Sample preparations: Male and female plants were collected from the bank of river Ravi, near Shahdara, Lahore, Pakistan. Inflorescence, leaves and stem of the two genders (male inflorescence: MI; female Inflorescence: FI; male leaves: ML; female leaves: FL; male stem: MS and female stem: FS) were separated manually. Distilled water-washed, shade-dried and finely ground plant material of each part was initially extracted in methanol. The methanolic residue was later suspended in distilled water in a separating funnel and subjected to partitioning in *n*-hexane, chloroform, ethyl acetate and 1-butanol successively. The resultant extract of each fraction was concentrated on rotary evaporator to a solid/semi solid residue. Standard solution of each residue (0.2 g/5 mL) was prepared using the same solvent in which it had been extracted. The solutions were refrigerated in amber-coloured bottles at 4 ºC until they were used in antioxidant assays. The solutions of standard antioxidants and other reagents were made either in double distilled water or methanol, depending upon their solubility.

Modified $ABTS^{\cdot+}$ decolourization assay protocol: The absorbance inhibition of the coloured $ABTS^{\cdot+}$ solution by sample/standard antioxidants forms the basis of the assay. ABTS assay protocol as developed by Re *et al.*[28] with some modifications[29], was followed for the antioxidant activity evaluation of plant extracts. For $ABTS^{\cdot+}$ stock solution ABTS, dissolved in double distilled water to a 7 mM concentration, was reacted with 2.45 mM potassium persulfate (final concentration). The solution was allowed to stand in the dark at room temperature for 12-16 h to form an intense bluish green solution. For antioxidative studies of the sample solutions, the working ABTS radical solution was obtained by diluting the stock solution with phosphate-buffered saline (pH 7.4) to an absorbance of 0.70 (± 0.02) at 734 nm and equilibrating at 30 ºC. The plant extracts were diluted in the respective solvents whenever needed. An aliquot (10 µL) of neat or diluted plant sample solution was mixed with 2.99 mL of the working $ABTS^{\cdot+}$ solution (A = 0.700 ± 0.020), the absorbance reading was taken at 30 ºC, exactly 1 min after initial mixing up to 8 min. Appropriate solvent blanks were run in each assay. The per cent inhibition of absorbance at 734 nm (% $I_{734\ nm}$) was calculated by the following formula and was plotted as a function of concentration or volume of standard or sample antioxidant solutions.

$$I_{734\ nm}\ (\%) = \left(1 - \frac{I_f}{I_o}\right) \times 100$$

where I_o and I_f are the absorbances of radical cation solution before and after the mixing of sample/standard antioxidant solutions, respectively. Each measurement was made in triplicate at each concentration level of sample and standard.

DPPH assay protocol[30]: The DPPH free radical scavenging effect of the plant extracts was demonstrated. The DPPH solution (2.99 mL, 25 mg/L), adjusted to A = 1.0 ± 0.01 at 517 nm with methanol, was mixed with 10 µL neat or diluted sample solution. The reaction progress of the mixture was monitored at 517 nm for 0.5 h. Upon reduction, the colour of the solution faded. A kinetic curve showing the scavenging of DPPH radical in terms of decrease in absorbance at 517 nm as a function of time (min) was plotted for each fraction of the plant sample.

Total phenolic content assay: The concentration of total phenolic phytochemical content was estimated using Folin-Ciocalteu's method[31]. Stock solution of gallic acid was made by dissolving 0.5 g gallic acid in 10 mL of ethanol in a 100 mL volumetric flask and diluted to volume with doubly distilled water. Sodium carbonate solution was prepared by dissolving 200 g of anhydrous sodium carbonate in 800 mL of doubly distilled water. After boiling and subsequent cooling of the solution, a few crystals of sodium carbonate were added. The solution was allowed to stand for 24 h, filtered and volume was raised to 1 L with doubly distilled water. To prepare a calibration curve, 0, 1, 2, 3, 5 and 10 mL of phenol stock solution were added into 100 mL volumetric flask separately and then diluted to volume with doubly distilled water. The resultant solutions contained concentrations of 0, 20, 40, 60, 80 and 100 mg/L gallic acid.

From each calibration solution and sample or blank, 40 µL were pipetted into separate cuvettes and to each 3.16 mL of double distilled water was added. Folin-Ciocalteu phenol

reagent (200 µL) was added and mixed well. After 8 min, 600 µL of sodium carbonate solution was mixed thoroughly in the solution. The solution was allowed to stand at 40 ºC for 0.5 h and absorbance of each solution was determined at 765 nm against the blank (without phenolic solution). A concentration *versus* absorbance linear graph was plotted. The concentration of total phenolic compounds of each fraction [as milligram of gallic acid equivalent (GAE)] was determined by using the standard gallic acid plot.

Total antioxidant activity: Total antioxidant activity of aqueous and organic extracts was determined according to reported method[32]. The solution, which contained 100 µL each of neat or diluted plant extract of both the plants in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). Each solution was then incubated in sealed bottles kept in dark at 37 ºC. The solution without added extract was used as blank, while the solutions containing 100 µL (50 µg/20 µL) of trolox was used as positive control. At intervals of 24 h during incubation, 0.1 mL of each solution was transferred to a beaker containing 3.7 mL of ethanol. After addition of 0.1 mL each of $FeCl_2$ (20 mM in 3.5 % HCl) and thiocyanate solution (30 %) to ethanolic sample, the solution was stirred for 1 min. The absorption values of the solutions measured at 500 nm were taken as lipid peroxidation values.

Iron(II) chelating activity: Ferrous ion (Fe^{2+}) chelation by plants/herbs extracts was estimated by the ferrozine assay[33]. An aliquot of sample (100 µL) was added to a solution of 0.05 mL of freshly prepared 2 mM $FeCl_2$. The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and total volume was adjusted to 4 mL with ethanol. The mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. The results were expressed as percentage of inhibition of ferrozine-Fe^{2+} complex formation. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

$$\text{Iron(II) chelating activity (\%)} = \left[\frac{(A_{Control} - A_{Sample})}{A_{Control}}\right] \times 100$$

where $A_{Control}$ is the absorbance of the control and A_{Sample} is the absorbance in presence of the sample.

RESULTS AND DISCUSSION

$ABTS^{\cdot+}$ decolourization assay: The basic principle underlying any decolourization antioxidant assay is the inhibition of absorbance of a coloured probe by sample antioxidants at a specific wavelength in a stipulated time period. A modified ABTS radical decolourization assay[29] involving a more stable ABTS radical cation formed in the presence of sodium acetate buffer (20 mM, pH 6.5) was employed to evaluate radical scavenging and antioxidant activity of the sample extracts. Standard and sample antioxidants that are able to transfer an electron scavenge the coloured ABTS radical solutions proportionate to their amount. The degree of colour scavenging depends both upon the nature and concentration of antioxidant sample. The organic extracts from different parts of *C. sativa* showed significant ABTS radical scavenging activity. Fig. 1 shows that antioxidant activity of different fractions of MS decreases in the order of 1-butanol > methanol > *n*-hexane > chloroform > ethyl acetate. The difference in the degree of radical scavenging was found much significant between the polar (1-butanol and methanol) and the relatively less polar fractions. This may be attributed to the relatively better solubility of the antioxidative components in the polar solvents. Same ABTS radical scavenging activity pattern with different degree of variations (not shown here) was also obtained for other parts of Cannabis plant. The greater steepness of the time dependent curves obtained for almost all the fractions shows that the radical-antioxidant reaction is fast and completes within 2.0-2.5 min. After 2.5 min the curves become almost parallel to the time axis with a very small change in absorbance indicating that the reaction has practically completed. Trolox equivalent antioxidant capacity (TEAC) values obtained for various extracts of different parts of *C. sativa* were calculated and are tabulated in Table-1. On the basis of the TEAC data given in Table-1, it is quite evident that 1-butanol followed by methanol and *n*-hexane is the most effective solvent for the extraction of antioxidant components of the extracts under analysis. Higher TEAC values of these extracts may be attributed to the polyphenols present in these samples[12-14,17-20].

Total phenolic contents: Antioxidant compounds including flavonoids, vitamins, carotenoids and other polyphenols are derived from plants, fruits and vegetables. These polyphenols especially the flavonoids, demonstrate multi-functional role. In the biological systems they hinder the enzyme-mediated generation of reactive oxygen species, capture free radicals and inhibit Fenton's reaction by chelating transition metals[34]. Since these compounds function through different mechanisms and their efficiency depends upon their nature and the test conditions, many researchers had suggested performing more than one kind of antioxidant assays to take into account the real picture of antioxidative ability of the compounds[35,36].

TABLE-1
TEAC VALUES (mM) OF VARIOUS EXTRACTS FROM DIFFERENT PARTS OF *C. sativa* BY A MODIFIED ABTS RADICAL CATION DECOLOURIZATION ASSAY

Fraction	TEAC (mM)					
	MI	FI	ML	FL	MS	FS
Methanol	85.65	46.81	41.26	72.41	51.57	15.86
1-Butanol	7.47	144.46	48.81	60.89	70.95	121.85
Ethyl acetate	1.66	30.56	20.23	16.73	14.48	3.72
Chloroform	12.63	7.94	1.69	7.62	15.39	1.47
n-Hexane	12.47	14.61	89.00	15.45	29.17	25.53

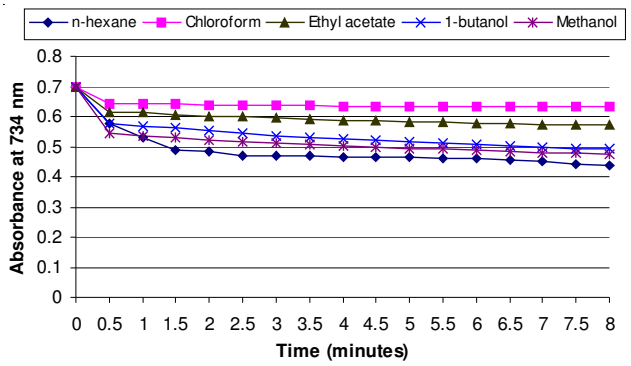

Fig. 1. Time dependent ABTS radical scavenging by different fractions of MS of *Cannabis sativa*. *n*-Hexane and ethyl acetate fractions are 10 times, chloroform and methanol fractions are 20 times and 1-butanol fraction is 30 times diluted in the respective solvent

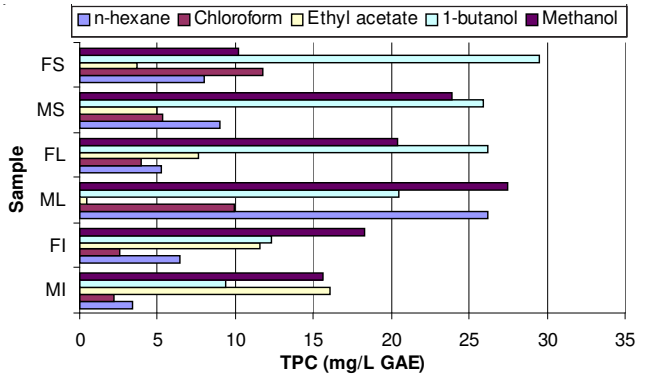

Fig. 2. Total phenolic contents (in terms of gallic acid equivalents (mg/L)) of various fractions of leaves stem and inflorescence of *Cannabis sativa*

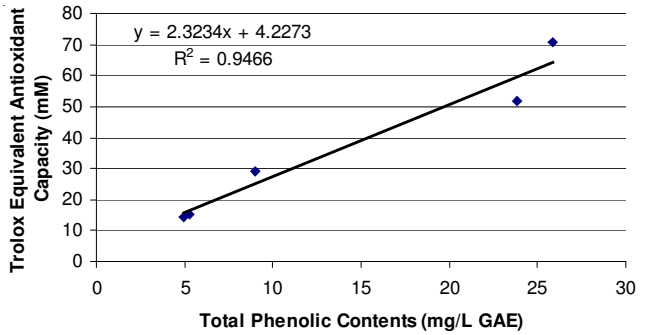

Fig. 3. A positive correlation between total phenolic contents and trolox equivalent antioxidant capacity of extracts from stem of male plant of *C. sativa*

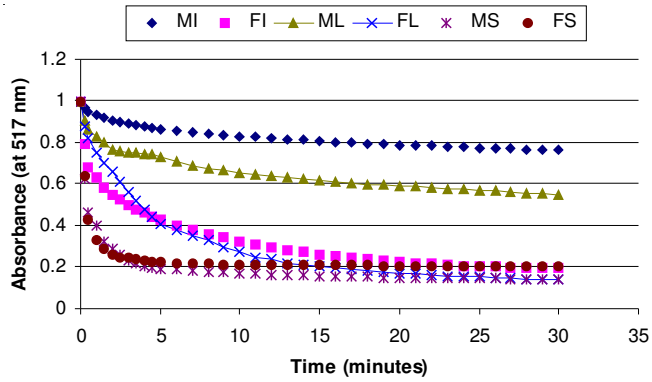

Fig. 4. Kinetics of scavenging of DPPH free radical of 1-butanol fraction of various parts of *Cannabis sativa*

The total phenolic contents (TPCs) of crude plant extracts are indicative of their cumulative or total antioxidant activity. The Folin-Ciocalteu phenol reagent is a mixture of phosphotungstic acid and phosphomolybdic acid and is used to obtain a crude estimate of the amount of phenolic present in an extract. Phenolic compounds undergo a non-specific reaction with FC reagent to form blue coloured complex species. Various reports have indicated the poor specificity[37,38] and variable response of the method to varying number of phenolic groups[38].

Phenolic compounds exhibit their antioxidant action by donating single election to the radicals. Total phenolic contents of the sample extracts were evaluated using the Folin-Ciocalteu's method. All the fractions of *C. sativa* showed a range of total phenolic content values. Phenolic compounds were found distributed amongst different fraction in the decreasing order of 1-butanol > methanol > *n*-hexane > ethyl acetate > chloroform (Fig. 2). This pattern is in agreement with the pattern obtained for TEAC values. High TEAC values obtained by using ABTS method may be accredited to high level of total phenolic compounds present in these samples. In case of MS a significant correlation ($R^2 = 0.9466$) was obtained between total phenolic contents and Trolox equivalent antioxidant capacity values (Fig. 3). But no such correlation could be established for other samples like MI, FI *etc*. These opposite trends could be justified on a number of counts. Firstly, total phenolics alone do not demonstrate the total antioxidant power of the sample. There may be many other chemical entities which lack hydroxyl moieties but still they work as bio-reductant and radical scavengers. Secondly, the synergistic interactions amongst chemical constituents of extracts may also contribute for non-significant correlation between total phenolics and TEAC levels[39,40].

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity: The kinetic curves obtained by plotting absorbance at 517 nm of DPPH solution against time for 1-butanol fraction of various extracts of *C. sativa* is shown in Fig. 4. The rate of scavenging of DPPH radical for various fractions reflected the presence of a diverse nature of antioxidative components in all the fractions of the three parts of the plant. Fig. 3 showed that MSB and MSL fractions of *C. sativa* contained the highest and MIB and MLB fractions the lowest levels of DPPH radical scavenging agents when studied for a period of 0.5 h. For 1-butanol fractions, various degree of steepness of the curves in the first 15-20 min demonstrated the order of the reaction in the decreasing order of MS > FS > FL > FI > ML > MI.

Lipid peroxidation inhibition activity using linoleic acid emulsion systems: Lipid peroxidation relates to oxidation of polyunsaturated fatty acids causing rancidity of food and pathological complications in living organisms[41,42]. Reactive oxygen species including hydroxyl ($OH^{\cdot}$), peroxyl radicals ($ROO^{\cdot}$) and the superoxide anion radicals ($O^{\cdot 2-}$), produced endogenously as a result of either metabolic processes or some exogenous factors, have been linked to different pathological conditions[43,44]. Natural antioxidants resist the production of ROS by inhibiting the lipid peroxidation[41,42].

In the present study, linoleic acid emulsion system was employed to evaluate lipid peroxidation inhibition activity of the extracts from *C. sativa*. Aerial oxidation of linoleic acid generates peroxyl radicals which have the ability to oxidize Fe^{2+}-Fe^{3+}. Thiocyanate ions (SCN^-) on reaction with these Fe^{3+} ions form a coloured complex which can be monitored at 500 nm spectrophotometrically. The sample solution tries to inhibit oxidation of linoleic acid and thus formation of peroxyl radicals. Thus low absorption values which are also called peroxidation values indicate high antioxidant activity of samples. Lipid peroxidation values of all the fractions were determined as a function of time. The results showed that all the fractions of both the genders of the plants have significant lipid peroxidation inhibition activity (Fig. 5).

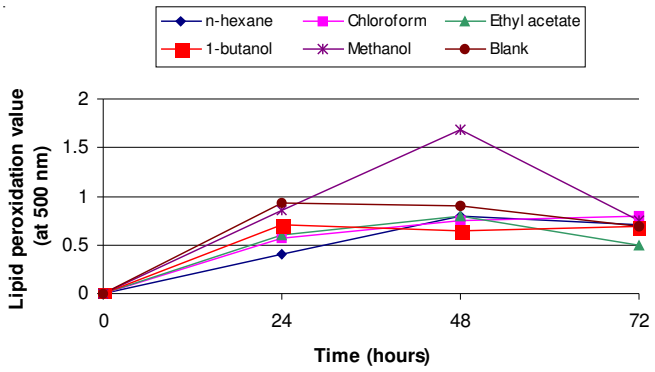

Fig. 5. Lipiid peroxidation values (at 500 nm) of various fractions of leaves of female plant of *Cannabis sativa*

Metal chelating activity the ferrozine activity: Transition metals especially iron(II) present in the biological systems act as pro-oxidant. Pro-oxidants facilitate the generation of potentially harmful species *in vivo*. Antioxidative compounds like polyphenoles, by chelating Fe(II), inhibit it to act as pro-oxidant. Ferrozine reacts with ferrous ions *in vitro* to form a coloured complex which can be monitored spectrophotometrically at 562 nm. In the presence of sample, ferrozine has to compete with polyphenolic compounds *etc*. (which are good Fe^{2+} chelators) for the formation of this complex. Thus a decrease in the absorbance at 562 nm occurs proportionate to the amount of Fe^{2+} chelating agents present in the sample. The results of inhibition of ferrozine-Fe^{2+} complex are expressed as percent chelating activity or percent bound iron. The per cent chelating activity for all the extracts was calculated and graphed (Fig. 6). One of the abojectives of the present study was to evaluate the chelating ability of organic extract of *C. sativa* against iron(II) generated *in vitro*. The per cent chelating activity ranged for different fractions ranged from 9.46-84.94 for ethyl acetate fraction of ML and methanol fraction of FL, respectively (Fig. 6). On the whole, methanol, 1-butanol and *n*-hexane fractions exhibited higher values of chelation. Phenolic compounds have been reported to be chelators of free metal ions. However, a poor correlation of ferrous ion chelating activity with total phenolic contents of all extracts was observed and this indicates that phenolic compounds might not be the main chelators of iron ions.

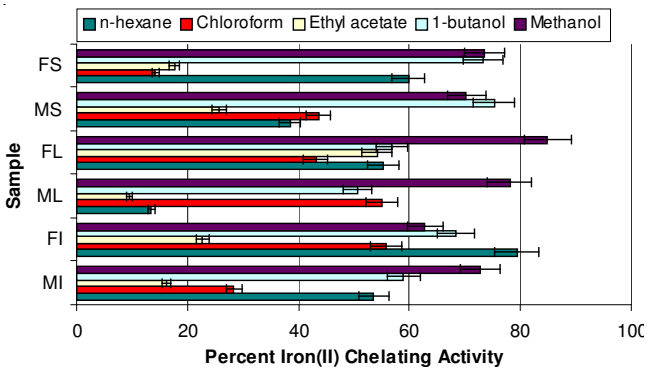

Fig. 6. Per cent iron(II) chelating activity of various fractions of inflorescence, leaves and stem of male and female plants of *Cannabis sativa*. Data represent mean ± standard deviation

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