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IDENTIFICATION OF ACTIVE COMPOUNDS USING GC–MS AND ANTIOXIDANT ACTIVITIES OF HIBISCUS CANNABINUS FLOWERS

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ABSTRACT

Natural antioxidants present in the plants scavenge harmful free radicals from our body. Antioxidants exert their mode of action by suppressing the formation of reactive oxygen species either by inhibition of enzymes or by chelating trace elements. Free radicals are the chemical species that have an unpaired electron and play very important role in human health and beneficial in combating against several diseases like cardiovascular disorders, lung damage, inflammation etc. These can damage cell membranes and other vital cell components, such as genetic material in the cell nucleus, and can inactivate enzymes. Damage to body cells and molecules by oxygen containing free radicals has been implicated in a wide variety of diseases. Antioxidants in different parts of plants such as ascorbic acid, vitamin E and phenol compounds possess the ability to reduce the oxidative damage associated with many diseases including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing. This study was aimed to evaluate *in vitro* antioxidant activities of *Hibiscus cannabinus* flowers. For *in vitro* antioxidant screening, DPPH, ABTS, hydroxyl radical scavenging, hydrogen peroxide scavenging activities and ferric reducing power models were used in this research. Also, the current work, analyses the chemical constituents *Hibiscus cannabinus* flowers using GC-MS.

KEYWORDS: *Hibiscus cannabinus* flowers, GCMS, Antioxidant, DPPH, ABTS

INTRODUCTION

Phytochemicals are biologically active plant chemicals that provide various health benefits.[1,2] They are naturally occurring bioactive substances that provide plants with particular defence mechanisms and protect them from various diseases. An important category of phytochemicals commonly present in plants has high antioxidant activity.[3,4] Phytochemical analyses are of paramount importance for the identification of new sources of therapeutically and industrially valuable compounds with medicinal significance and for the best and most judicious use of naturally available materials.[5] Flavonoid and phenolic compounds, which exhibit a broad range of biological activities, are known to be more abundant than flavonoid monomers in plants.[2] The wide use of plants as food, food additives and drug has increased the number of researches investigating the phytochemicals and biological activity of these sources.[6,7] The medicinal plants were used for health care because they contain aromatic components of high therapeutic potential. Especially, essential oils and fatty acids are widely used in medicine, as flavouring additives in the food industry, and as fragrances in the cosmetic industry.[8,9]

Hibiscus cannabinus (Kenaf), an annual herbaceous crop of the Malvaceae family, is a short-day plant. *Hibiscus cannabinus*, a valuable medicinal crop originated from Africa, is contained various functional compounds. The *Hibiscus cannabinus* leaf was applied to Guinea worms and the stem bark has been used for anaemia in Africa.[10] In ayurvedic medicine, the kenaf leaves are used for bilious, blood, diabetes, coughs and throat disorders.[1,10,2] The flower juice is used for biliousness.[10] The seeds are also consumed to weight increase and bruises.[11] The *Hibiscus cannabinus* leaf and seed contains a variety of different compounds, including phenolic compounds, flavonoids, essential oils, and fatty acids.[12-14] *Hibiscus cannabinus* has been reported to exhibit properties associated with anodynes, aperitifs, aphrodisiacs, anti-inflammatory medications, and antioxidants for leaf and seed. It has also been related to weight gain, anemia, and fatigue.[8,10] Phenyl-propanoids, which are abundant in the *Hibiscus cannabinus* leaf, are important for these beneficial health effects.[2] Various biologically active compounds have been reported in the kenaf seed, including omega-3 fatty acids, phenolic compounds, and sterols.[10] However, studies on the phytochemical of different parts were little known. A selection of the best solvents for the extraction of chemical compounds from plant materials is important to improving the efficiency of extraction yield.[15] Therefore, it is essential to determine the solvents on the extraction of functional compounds and antioxidant

properties of *Hibiscus cnnabinus* to select an optimal solvent for the extraction of the bioactive compounds from the different parts of the *Hibiscus cannabinus* plant. This study analyzed the antioxidant activities in the flowers of *Hibiscus cannabinus*. This is the first report on the phytochemical composition of the flowers of the *Hibiscus cannabinus* plant to identify novel, potentially environmentally friendly natural products that would be useful in the food industry. Additionally, the antioxidant activity was investigated.

MATERIALS AND METHODS

Plant material: The *Hibiscus cannabinus* flowers (Fig.1) were collected from Thanjavur Tamil Nadu. About 1.0 kg of shade-dried coarse powder of the flower material was extracted with 80%v/v aqueous ethanol by maceration at room temperature for 72 h. After the completion of each extraction, the extracts were filtered, concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-50°C). The residues were stored in a vacuum desiccator for further use.

Fig. 1: *Hibiscus cannabinus* flowers.

GC–MS: The ethanolic extract was subjected to GC-MS analysis on the instrument GC and MS JEOL GC mate equipped with secondary electron multiplier. JEOL GCMATE II GC-MS with with Data system is a high resolution, double focusing instrument. Maximum resolution: 6000 Maximum calibrated mass: 1500 Daltons. Source options: Electron impact (EI); Chemical ionization (CI) (Agilent Technologies 6890N Network GC system for gas chromatography). The column (HP5) was fused silica 50 m x 0.25 mm I.D. Analysis conditions were 20 min. at 100°C, 3 min at 235°C for column temperature, 240°C for injector temperature, helium was the carrier gas and split ratio was 5:4. The sample (1 µl) was evaporated in a split less injector at 300°C. Run time was 22 min. Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST08 and Wiley08 library. Identification of components was based on comparison of their mass spectra.

Antioxidant activity assay

DPPH radical scavenging activity

Various concentrations (20, 40, 60, 80 & 100µg/mL) of extracts were mixed with 3.0 mL of methanolic solution containing DPPH radical (6×10^{-5} mol/L). The mixture was shaken vigorously and left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by recording the absorbance at 517 nm using UV–Vis spectrophotometer. DPPH radical-scavenging activity was calculated by the following equation:

DPPH radical scavenging activity % = $(A_c - A_s)/A_c$ X 100

Where A_c is the absorbance without samples and As the absorbance in the presence of the samples. A lower absorbance of the reaction mixture indicated a higher DPPH radical-scavenging activity.[16]

Hydrogen peroxide radical scavenging activity

Hydrogen peroxide solution (1 mM/L) was prepared with 50 mM phosphate buffer (pH 7.4). Different concentrations (20–100µg) of the extract (1 mL) were allowed to react with 0.6 mL of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Hydrogen peroxide scavenging activity was calculated according to the following equation:

Hydrogen peroxide scavenging activity % = $(A_c - A_s)/A_c$ X 100

Where A_c is the absorbance without samples and A_s is the absorbance in the presence of the samples.

Ferric reducing power

Exactly 1 mL of the extract was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50°C for 30 min. Afterwards, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of the upper layer was pipetted out and mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride (0.1%) was added. The absorbance was measured at 700 nm using a UV-Visible Spectro-photometer. The intensity of reducing power is directly proportional to the absorbance of the reaction mixture.[17]

ABTS radical scavenging activity

ABTS radical scavenging activity was estimated by the method of Gülçinet et al. 2010.[18] The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persuphate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for overnight at room temperature in the dark. The solution was then diluted by mixing 1 mL of ABTS solution with 60 mL

ethanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm. Fresh ABTS solution was prepared for each assay. Different concentrations (20–100µg) of the extracts (1 mL) were allowed to react with 1 mL of the ABTS solution and the absorbance was measured at 734 nm after 7 min using a UV-Visible Spectrophotometer. ABTS radical scavenging activity was calculated according to the following equation:

ABTS radicalscavenging activity % = $(A_c - A_s)/A_c$ X 100

Where Ac is the absorbance without samples and As the absorbance in the presence of the samples.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the extracts was carried out by the method of Halliwell and Gutteridge 1981.[19] Exactly, 0.2 mL of the extract was added with 1.0 mL of EDTA solution (0.13 g of ferrous ammonium sulphate and 0.26 g of EDTA were dissolved in 100 mL of water) and mixed with 1.0 mL of DMSO (0.85%) in 0.1 M phosphate buffer (pH 7.4) to initiate the reaction followed by the addition of 0.5 mL of 0.22% ascorbic acid. The reaction mixture was kept in a water bath at 90°C for 15 min and the reaction was terminated by adding 1.0 mL of ice-cold 17.5% trichloroacetic acid. Further 3.0 mL of Nash reagent (75 g of ammonium acetate, 3.0 mL of glacial acetic acid and 2.0 mL of acetyl acetone in 1.0 L of water) was added to all the test tubes and incubated for 15 min for colour development. The absorbance was observed at 412 nm. The reaction mixture without ascorbic acid served as control. The ability to scavenge hydroxyl radical was calculated by the following equation:

Hydroxyl radical scavenging activity % = $(A_c - A_e)/A_c$ X 100

Where Ac is the absorbance without samples and Ae the absorbance in the presence of the samples. Vitamin C was used as a standard.

RESULTS AND DISCUSSION

Chemical composition by GC-MS analysis: Interpretation on mass spectrum GC–MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. Seven compounds were identified in *H. Cannabinus* flower extract by GC– MS analysis was shown in Fig.2. The active principles with their retention time (RT) and concentration (%) were taken into account.

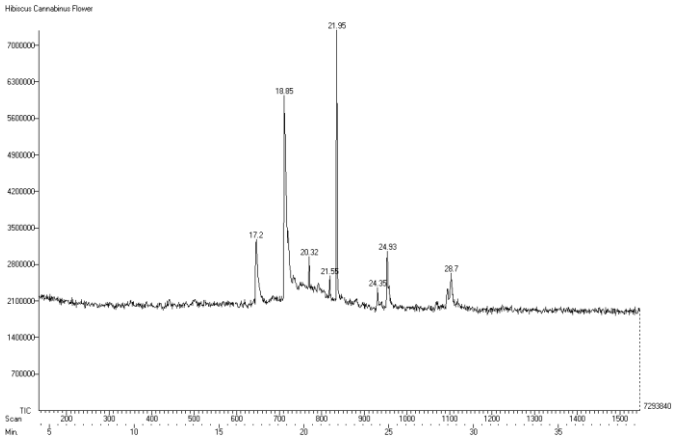

Fig. 2: GC-MS chromatogram of *Hibiscus cannabinus* flower extract.

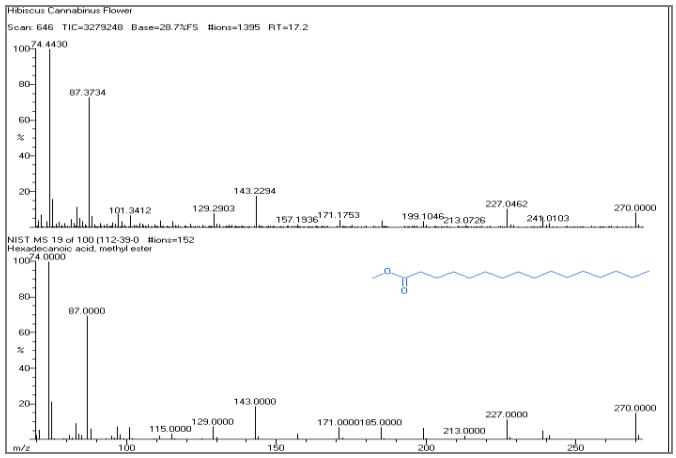

Fig. 3: Mass spectrum of Hexadecanoic acid, methyl ester.

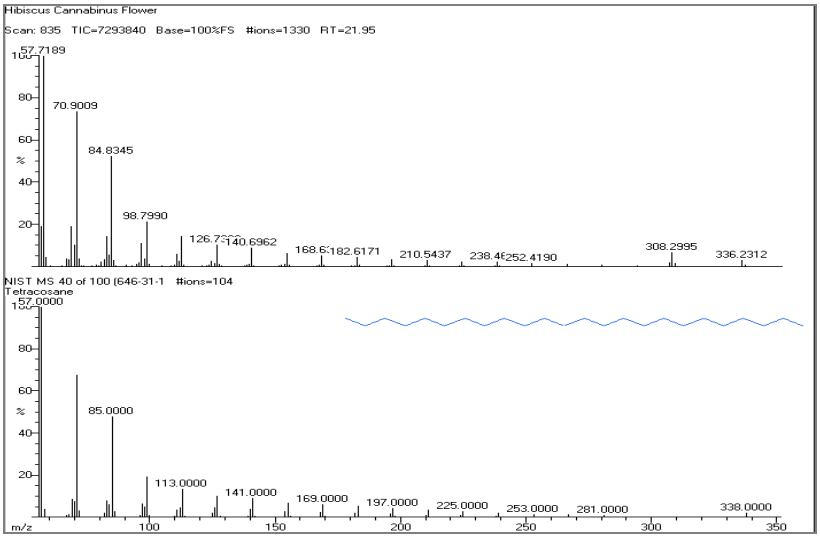

Fig. 4: Mass spectrum of Tetracosane.

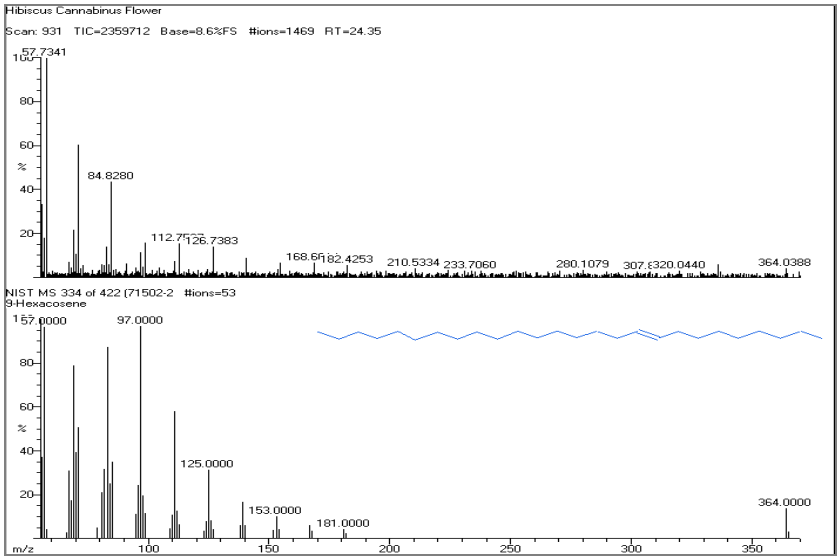

Fig. 5: Mass spectrum of 9-Hexacosane.

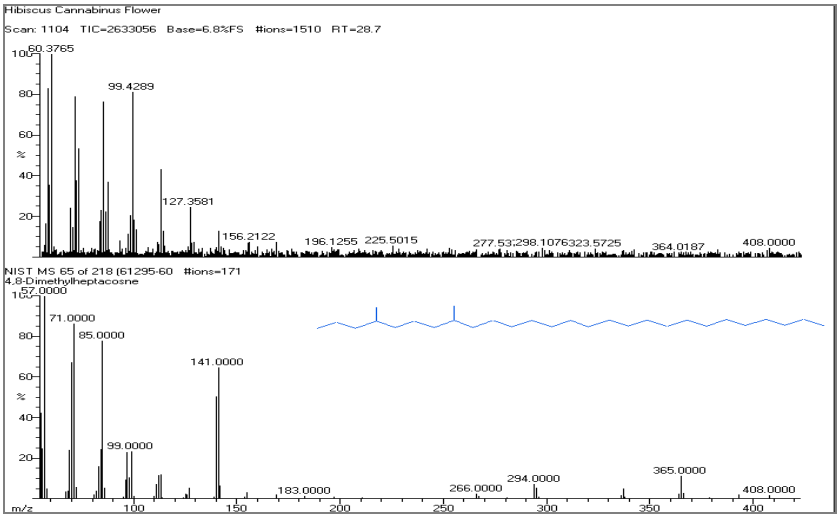

Fig. 6: Mass spectrum of 4,8-Dimethyl heptacosane.

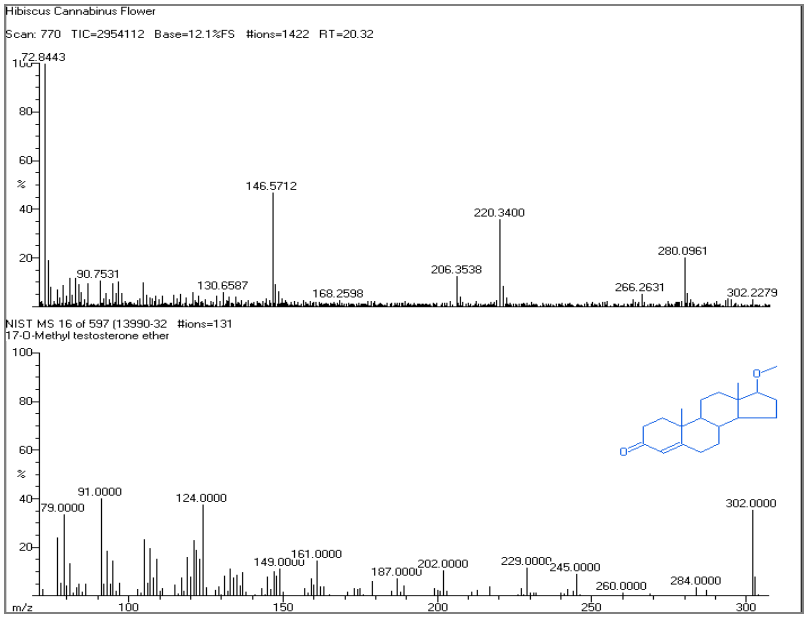

Fig. 7: Mass spectrum of 17-O-methyl testosterone ether.

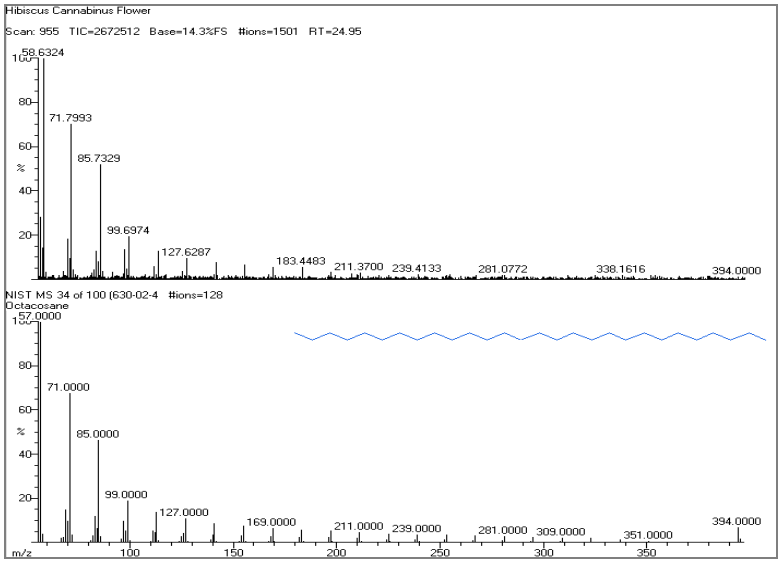

Fig. 8: Mass spectrum of Octacosane.

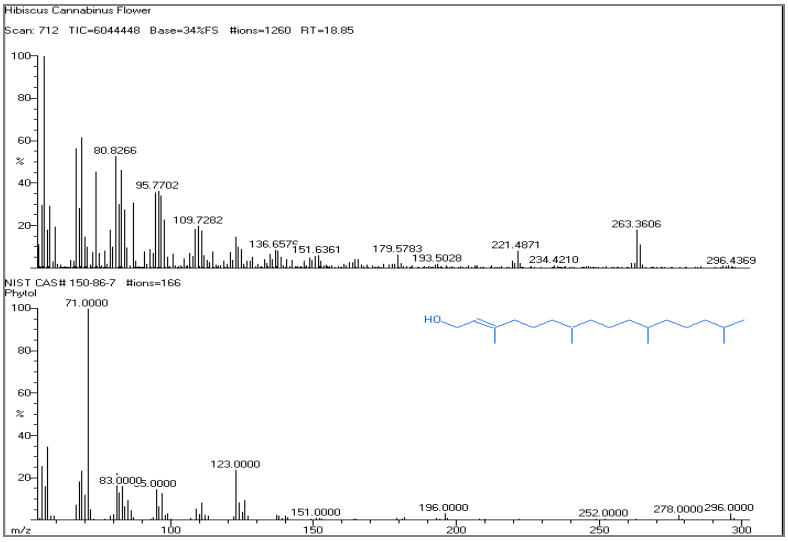

Fig. 9: Mass spectrum of Phytol.

Table 2: Phytoconstituents identified in alcohol extract of *H. Cannabinus* flowers.

No.	Base %	RT	Name of the compound
1	28.7	17.2	Hexadecanoic acid, methyl ester
2	34	18.85	Phytol
3	12.1	20.32	17-O-methyl testosterone ether
4	100	21.95	Tetracosane
5	8.6	24.35	9-Hexacosane
6	14.3	24.95	Octacosane
7	6.8	28.7	4,8-Dimethyl heptacosane

The flower extract contained seven compounds among them, phytol (RT 18.85) is having anticancer, antioxidant, anti-inflammatory, antitumor, antimicrobial, diuretic, and chemopreventive properties and used in vaccine formulations.[20] Phytol also found to be effective at different stages of the arthritis. It was found to give food as well as preventive and therapeutic results against arthritis. The results show that, reactive oxygen species – promoting substances such as phytol constitute a promising novel class of pharmaceuticals for the treatment of rheumatic arthritis and possibly other chromic inflammatory diseases.[21] Phytol reported to posses diuretic, antimicrobial, anti-inflammatory and anticancer activity.[22]

Octacosane (RT 24.95) found to be Mosquitocidal activity. Tetracosane (RT 21.95) acts as anti-corrosive and antioxidant. Hexadecanoic acid methyl ester (RT 17.2) seem to have the ability to decrease blood cholesterol, inhibits the cyclooxygenase II enzyms and, thus, produce a selective anti-inflammatory action.[23]

Antioxidant activity assay

DPPH radical scavenging activity

The radical-scavenging activity of the flower extract was estimated by comparing the percentage inhibition of formation of DPPH radicals with that of vitamin C. Flower extract showed moderate antioxidant activity when compared with Vitamin C. The DPPH radical scavenging activity of flower extract increased with increasing the concentration (Fig.10). Natural antioxidants those are present in medicinal plants which are responsible for inhibiting the harmful consequences of oxidative stress. Many plants extract exhibit efficient antioxidant proper-ties due to their phytoconstituents, including phenolics. This method has been extensively used for screening antioxidants, such as polyphenols. The antioxidant effectiveness in natural sources has been reported to be mostly due to phenolic compounds. Phenolic compounds may contribute directly to antioxidative effect of the extracts. The free radical scavenging activity of flower extract was confirmed in the present investigation. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecules. This method has been extensively used for screening antioxidants, such as polyphenols. DPPH radical is scavenged by poly-phenols through donation of hydrogen, forming the reduced form of DPPH. Then the colour changes from purple to yellow after reduction, which can be quantified by its decrease absorbance at wavelength 517 nm.[24]

The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecule and radical progresses, results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow. These results revealed that the *H.cannabinus* flower extract is free radical inhibitor or scavenger acting possibly as primary antioxidants.

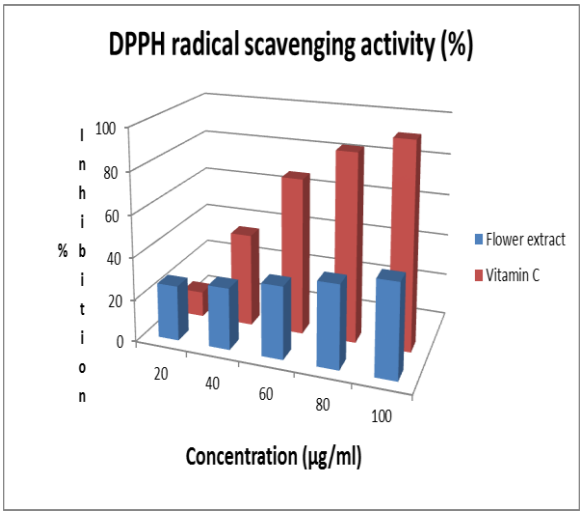

Fig. 10: DPPH radical scavenging activity (%).

Hydroxyl radical scavenging activity

Hydroxyl radical inhibition of *H.cannabinu*s flower extractwas investigated and these results are shown as relative activity against the standard (Vitamin C). Hydroxyl radical scavenging activity of flower extract is presented in Fig.11. Dose-dependent hydroxyl radical scavenging activity reveals that, *H.cannabinus* flower extract have potent hydroxyl radical scavengers, acting possibly as primary antioxidants. Hydroxyl radical is an extremely reactive free radical formed in biological system and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule, proteins, DNA, unsaturated fatty acids and lipids in almost every biological membranes found in living cells.[25]

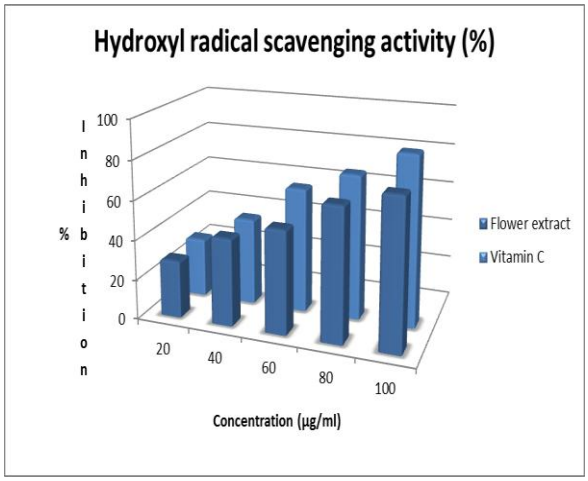

Fig. 11: Hydroxyl radical scavenging activity (%).

ABTS Radical scavenging activity

The results clearly imply that the flower extract of *H. cannabinus* inhibit ABTS radical or scavenge the radical in a dose dependent manner (Fig.12). ABTS radical is generated from oxidation of ABTS by potassium persulphate, is a good tool for determining the antioxidant activity of hydrogen-donating and chain breaking antioxidant.[26] This assay is applicable for both lipophilic and hydrophilic antioxidants. The radical-scavenging activity of the flower extract of *H. cannabinus* was estimated by comparing the percentage inhibition of formation of ABTS radicals with that of vitamin C. These extracts exhibited the highest radical-scavenging activities when reacted with the ABTS radicals.

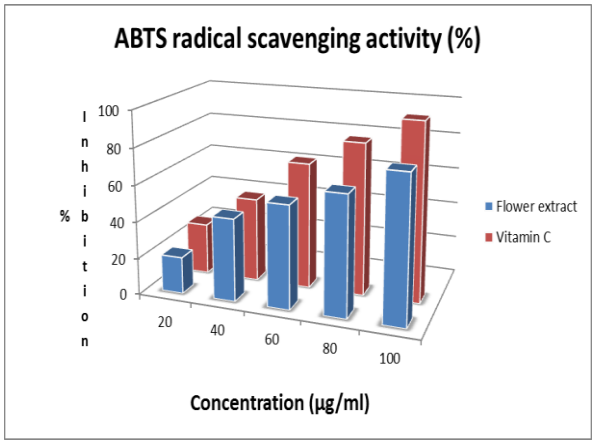

Fig. 12: ABTS radical scavenging activity (%).

Hydrogen peroxide radical scavenging activity

The hydrogen peroxide radical-scavenging activity of the flower extract of *H.cannabinus* was estimated by comparing the percentage inhibition of formation of peroxyl radicals with that of vitamin C. Hydrogen peroxide scavenging activity of flower extract presented in Fig.13. The results showed that flower extract *H.cannabinus* was highly potent in neutralizing hydrogen peroxide radicals. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. The result showed that *H. cannabinus* extract have an effective H_2O_2 scavenging activity.

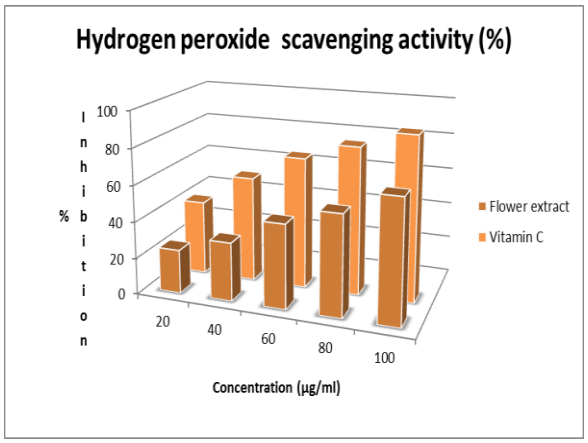

Fig.13: Hydrogen peroxide scavenging activity (%).

Ferric reducing power

In the reducing power assay, the presence of antioxidants in the extract of *H.cannabinus* would result in the reduction of Fe^{3+}/ferricyanide complex to its form. The reducing power of compound may serve as a significant indicator of its potential antioxidant activity.[27] The ferric reducing power of the flower extract of *H.cannabinus* was determined by comparing with that of vitamin C. The increased absorbance values of the extracts at 700 nm indicate an increase in reductive ability. Absorbance values flower extract was presented in Fig.14. Ferric reducing power increasing the absorbance values. The reducing power of ascorbic acid was found to be significantly higher than those of flower extract. In this assay, the yellow colour of the test solution was changed to various shades of green and blue depending on the reducing power of each compound. It was found that the reducing power of the extracts increased with the increase of their concentrations. This data imply that these extracts have significant ability to react with free radicals to convert them into more stable nonreactive species and to terminate radical chain reaction.

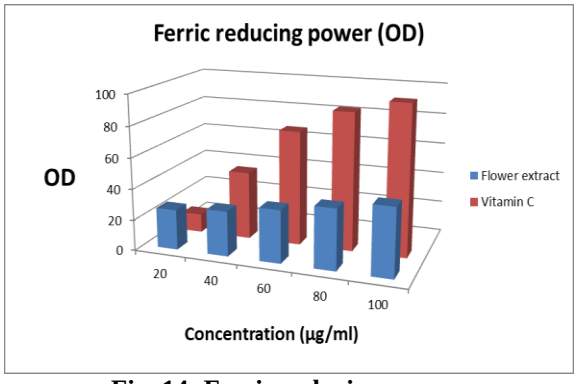

Fig. 14: Ferric reducing power.

CONCLUSION

Numerous methods are available to evaluate of antioxidant activity. For *in vitro* antioxidant screening,

DPPH, ABTS, hydroxyl radical scavenging, hydrogen peroxide scavenging activities and ferric reducing power are most commonly used. However, the total antioxidant activity of an antioxidant cannot be evaluated by using one single method, due to oxidative processes. Therefore, at least two methods should be employed in order to evaluate the total antioxidant activity. Present study was undertaken to demonstrate the antioxidant capacity of flower extract of *H. Cannabinus* by *in-vitro* methods. In the present study, seven compounds have been identified from ethanol extract of the flowers of *H.cannabinus* by GC-MS analysis. The presence of various bioactive compounds justifies the use of the leaf for various ailments by traditional practitioners. So it is recommended as a plant of phytopharmaceutical importance. However further studies will need to be undertaken to ascertain fully its bioactivity.

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