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QUALITY CONTROL AND IN VITRO ANTIOXIDANT ACTIVITY ANALYSIS OF ALCOHOLIC EXTRACT OF CROCUS SATIVUS

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ABSTRACT

The stigma of saffron, or Crocus sativus, has been used extensively as a spice, medicinal plant, and food ingredient in Mediterranean and Subtropical nations. Finding novel sources of natural antioxidants that are safe for the food sector has received attention lately. This study was conducted to determine the antioxidant activity of spices, including their total phenol and flavonoid content, DPPH, SOD, catalase, and total antioxidant activity. The antioxidant activities of spices are mostly due to their phenolic and flavonoid constituents. This study's goal is to determine the plant sample's antioxidant potential and standardise its quality through quality control analysis. The experimental study's findings demonstrated that the sample's quality was satisfactory and below the Ayurvedic Pharmacopoeia India reference level. Every necessary secondary metabolite is present in the plant sample. Saffron stigma has antioxidant action, according to an examination of its in vitro antioxidant activity. At half-maximal inhibitory concentration (IC50), the alcoholic extract of saffron stigma exhibited greater free radical scavenging action. Scavenging of DPPH determinate free radical scavenging capacity to inhibit lipid peroxidation is obtained 8.597 mg/ml which shows strong antioxidant activity. Total antioxidant activity, SOD and catalase activity in terms of IC50 value obtained from result are 18.97122mg/ml, 10.17387mg/ml and 7.564 mg/ml respectively. Results of this study revealed the strong antioxidant activity of the alcoholic extract of plant sample.

INTRODUCTION

Crocus sativus, usually known as saffron crocus, is a perennial flowering plant belonging to the Iridaceae family. It is best known for producing saffron, the highly prized spice derived

from the stigma of its flowers. Native to the Mediterranean region, this plant thrives in well-drained soil and prefers a sunny climate.[1]

Crocus sativus blooms in the fall, producing beautiful purple flowers that emerge from corms. Each flower has three vibrant red stigmas, which are harvested and dried to create saffron. Beyond its culinary uses, saffron is also celebrated for its potential health benefits and has been used in traditional medicine for centuries.[2] Crocus sativus, or saffron, is rich in antioxidants, which play a crucial role in protecting the body from oxidative stress caused by free radicals. Saffron contains several potent antioxidants, including crocin, crocetin, and safranal. These compounds help neutralize free radicals and reduce oxidative damage to cells. These antioxidants also contribute to its anti-inflammatory effects, potentially lowering the risk of chronic diseases linked to inflammation, help to improve heart health by preventing oxidative stress in blood vessels, which can reduce the risk of heart disease. This is very useful to protect brain cells from damage, potentially benefiting cognitive function and reducing the risk of neurodegenerative diseases.[3] The antioxidant activity of this plant has been the subject of minimal study. Therefore, the purpose of the study described here was to determine whether saffron stigmas, a common dietary spice, have antioxidant potential. Saffron stigmas shown antioxidant activity in one investigation, and methanol seemed to be the most effective solvent for removing the active ingredients.[4] Of these, gallic acid and pyrogallol may have contributed to the stigma's antioxidant qualities. Oxidant-antioxidant imbalance in the human body increases cell death and DNA damage, which can result in mutations and eventually lead to the development of chronic diseases like cancer. It also compromises immunity and cell activities.[5] Food additives and composition are important factors in supplying the body with the antioxidants it needs. Spices have long been used to enhance the flavour and taste of food preparations, but they are also widely used as dietary supplements that include antioxidants. According to reports, spices include bioactive chemicals that give food antibacterial, preservative, and antioxidant qualities. Numerous studies have demonstrated the antioxidant properties of spices that contain phenolic and flavonoid components. There has also been evidence of a positive linear relationship between the antioxidant capacity of spices and phenolic, flavonoids components.[6]

For many years, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), two synthetic antioxidants, have been employed extensively to slow down lipid oxidation. Scientists are now concerned about the safety of employing these artificial antioxidants in the

food sector, which has sparked a current search for natural antioxidants.[7] Because of this, a lot of spices have been tested for their antioxidative qualities before being used in food. Antioxidants are a group of compounds that help shield biological systems against the damaging effects of oxidative processes on macromolecules like DNA, proteins, lipids, and carbohydrates. Many of those compounds are natural compounds produced from plants that help prevent and treat diseases caused by reactive oxygen species. The ability of plant antioxidants to scavenge free radicals explains this protection. Scientists, food producers, and consumers are all very interested in the role antioxidants play in preserving health and preventing cancer and coronary heart disease. As a result, there should be a greater push to identify the antioxidant qualities of spice or herb plants.[8]

MATERIAL

Crocus sativus stigma is used in the current investigation to determine the pharmacognosy profile and antioxidant capability as per in vitro methodology. The plant sample is obtained from the GMP-certified Ayurvedic manufacturer Bilwal Medchem and Research Laboratory Private Limited, Reengus, Rajasthan, and authenticated for the same.

Reagents and chemicals

Methanol, HCl, NaOH, potassium ferrocyanide, glacial acetic acid, acetic acid, sodium nitroprusside, sulphuric acid, potassium chromate, toluene, and other necessary compounds Nitric acid, hypophosphorous acid, potassium iodide, potassium thiocynate, and carbon tetrachloride Mayer's reagent, PbSo4, ammonia solution, Ferric chloride, barium chloride, and Benzene acid perchlorica, Ammonium chloride, ethanol, sodium sulphide, acetone, potassium bismuth-iodide, sodium potassium tartarate, anisaldehyde, Chloroform Molisch's reagent, Millon reagent, solution of iodine, Pyridine, Seliwanoff's reagent, ninhydrin, Sulphate of copper, potassium hydroxide, Dragandrof reagents were acquired from Avantor, Merck, and HiMedia firms. These included sodium sulphate, sodium carbonate, aluminium chloride, Tris, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and Folin-Ciocalteu reagent.

METHODOLOGY

PHYSICOCHEMICAL ANALYSIS

- **LOSS ON DRYING**

After being weighed at about 10 g, the test sample that had never been dried previously was placed in a tared evaporating dish. I dried it for five hours at 105°C in a hot air oven. Until there is less than a 0.25% difference between two consecutive weight readings, drying and

weighing should be done every hour. After a 30-minute drying and cooling period in a desiccator, the weight was considered constant if there was a fluctuation of no more than 0.01 g between two subsequent weigh-ins. Determine the percentage of the specimen under evaluation that was lost in relation to 10 grammes.

- **Alcoholic Extractive Value**

Put 5 g of the coarsely powdered, air-dried test sample in a closed flask and macerate it with 100 ml of alcohol for 24 hours. After six hours of frequent shaking, let the mixture sit for the remaining eighteen hours. Filter as quickly as you can, being careful not to lose solvent. The filtrate should then be weighed after 25 millilitres of it have been evaporated in a shallow dish with a flat bottom until its weight remains consistent. 105°C was the drying temperature. With reference to the dried test sample, determine the extractive percentage of alcohol. By using methanol instead of alcohol, the methanol-soluble extractive was discovered.

- **Aqueous Extractive Value**

In a closed flask, macerate 5 g of the coarsely powdered, air-dried test sample with 100 ml of distilled water for 24 hours. For the first six hours, shake the mixture often; for the next eighteen hours, leave it alone. As quickly as you can, filter, being careful not to lose solvent. Next, in a shallow dish with a flat bottom, evaporate 25 millilitres of the filtrate until its weight remains constant, and then weigh it. dried at 105 degrees Celsius. Determine the extractive percentage that dissolves in water in relation to the dried test sample.

- **Total ash**

To remove carbon from the sample, burn 2 to 3 grammes of the powdered material in a silica crucible at a maximum temperature of 600°C. Weigh it once it has cooled. The burned mass was extinguished, the residue was gathered on ashless filter paper, the residue and filter paper were burned together, the filtrate was added, the evaporated material was dried, and the mixture was ignited at a temperature not to exceed 600°C in case carbon-free ash could not be produced using this method. Determine the percentage of dry ash in the test sample.

- **Acid-insoluble ash**

After adding all of the ash to the crucible, 25 millilitres of diluted hydrochloric acid were added. Whatman 41 ashless filter paper were used to collect the insoluble particles, and the filtrate was then cleaned in hot water until its pH was neutral. The filter paper containing the insoluble material was moved into the original crucible, dried on a hot plate, and then burned

until its weight remained constant. After letting the residue cool in the appropriate desiccator for half an hour, the residue was promptly weighed. Determine the percentage of acid-insoluble ash in the reference dry test sample.

- **Water-Soluble Ash**

Added 25 millilitres of distilled water to the crucible after adding all of the ash. Whatman 41 ashless filter paper were used to collect the insoluble particles, and the filtrate was brought to a pH of neutral by washing it in hot water. It was burned until it attained a steady weight after the filter paper containing the insoluble material was put back into the original crucible and allowed to dry on a hot plate. After letting the residue cool for half an hour in the appropriate desiccator, I weighed it right away. Determine the water-soluble ash percentage of the reference dried test sample.

Analysis of Primary and Secondary Metabolite Qualitatively

The major (carbohydrate, protein, and amino acid) and secondary (alkaloids, glycosides, tannin, saponin, and phenolic substances) metabolites are identified by phytochemical testing. Test samples were examined for primary and secondary metabolites using the alcoholic extracts.

• **Molisch's Test:** One millilitre of concentrated H2SO4 was poured out the side of the test tube and let to stand for a minute after two millilitres of test solution and two millilitres of Molisch's reagent were added, and the tube was gently shaken. The presence of carbohydrates was shown by a purple ring at the intersection of the two layers.

• **Benedict's test:** This method, which mostly used sodium hydroxide and copper sulphate, reduced sugars. One millilitre of Benedict's solution and four millilitres of the drug's aqueous solution were mixed together and heated to nearly boiling. The synthesis of cuprous oxide in the test solution produced colours like green, yellow, orange, red, or brown as the quantity of simple sugar increased.

• **Fehling solution test:** This test, which uses two in-situ blended solutions, is typically used to reduce blood sugar levels. Fehling solution B is made up of sodium potassium tartrate, while Fehling solution A contains 0.5% copper sulphate. One millilitre each of Fehling A and Fehling B solutions were blended, followed by the addition of two millilitres of the drug's aqueous solution. After that, the mixture was boiled for five to ten minutes in a water bath.

• **Dragondroff's Reagent:** In a test tube, two millilitres of the test solution and two millilitres of Dragondroff's reagent—bismuth subnitrate and potassium iodide solution—were added. The ability of the components to form an orange precipitate indicated the presence of alkaloids.

• **Wagner Test:** When a few drops of Wagner's reagent (diluted iodine solution) were added to two millilitres of test solution, a reddish-brown precipitate formed, indicating the presence of alkaloids.

• **Hager test:** A saturated aqueous solution was used to dissolve picric acid. The presence of alkaloids was shown by the orange-yellow precipitate that developed after the test filtrate was treated with this reagent.

• **Ninhydrin test:** This test was used to determine which proteins contained alpha-amino acids and free amino groups. The creation of a combination between two ninhydrin molecules and the nitrogen of free amino acids is what gives it its distinctive deep blue or light blue hue.

• **Biuret test:** Five milligrammes of residue, one millilitre of 4% sodium hydroxide solution, and a drop of 1% copper sulphate solution were added to the mixture. The appearance of a violet or pink tinge suggested the presence of proteins.

• **Xanthoprotic test:** Two millilitres of water and five millilitres of strong nitric acid were used to treat a small amount of the test material. The appearance of a yellow tint indicated the presence of proteins.

• **Foam test:** A small amount of the sample, a small amount of water, and sodium bicarbonate were put in a test tube, and the combination was agitated briskly. It was possible to identify saponins by their consistent, recognisable, honeycomb-like foam.

• **Borntragor's Test:** One millilitre of benzene and half a millilitre of diluted ammonia solution were combined with the ethanolic extract. The result was a reddish-pink colour that showed glycoside was present.

• **Phenolic compound test**: After heating the sample extract in water, two millilitres of ferric chloride solution were added. The liquid was then allowed to be watched to see whether any blue or green tint formed, which would indicate the presence of phenolic compounds.

• **Salkoweski reaction:** two millilitres of chloroform, five milligrammes of extract, and two millilitres of strong sulphuric acid were added from the test tube's side. A few minutes were spent shaking the test tube. Redness was a sign of the presence of steroids.

• **Lead acetate**: A 10% w/v solution of basic lead acetate in distilled water was combined with the test filtrate. The presence of tannins was indicated by the precipitate formation.

• **Potassium dichromate:** For this test, the filtrate was subjected to a potassium dichromate solution. The black colour suggested the presence of tannins.[9]

In vitro antioxidant activity analysis

Total Phenol Content

The concentration of all phenolic components in the methanol extract was determined using the Folin-Ciocalteu method. Each tube was filled with 750 µL of the Folin-Ciocalteu reagent. 100 µL of the extracts were then added to each tube, vortexed, and allowed to sit at 25°C for five minutes. 750 µL of sodium carbonate was then added to the tubes, and the solutions were kept at 25°C for 40 minutes. Following the determination of the absorbance at 725 nm, a standard curve was drawn. The results were expressed in gallic acid equivalent milligrammes per 100 grammes (mg GAE/100 g) of dry extract.[10]

Total Flavonoids Content

The content of all flavonoids was determined by colorimetric analysis using aluminium chloride. Initially, a 2% ethanol-based aluminium chloride solution was prepared. Then, 500 µL of the aluminium chloride solution (2%) and the test sample's methanol extract (10 mg/ml) at various concentrations were added to different tubes. After vortexing, the samples were stored for an hour at 25 °C. Absorbance measurements were performed at 420 nm, and reference curves for flavonoids were created using quercetin. According to the results, each 100 grammes of dry extract contained milligrammes of quercetin equivalent (mg QE/100 g).[11]

Total Antioxidant Capacity

The total antioxidant capacity was determined using the phosphomolybdate procedure. Each tube was filled with 0.1 millilitre of the extract and 1 millilitre of the phosphomolybdate reagent. The resulting solutions were vortexed and then brought to a boil for 90 minutes. Absorbance measurements were performed at 695 nm, and an antioxidant reference curve

was created using ascorbic acid. The results were expressed as the equivalent of ascorbic acid per gramme (AAE/g) of dry extract.

DPPH Assay

Before the tubes were vortexed, 0.8 mL of buffer, 0.2 mL of extracts (10, 20, 40, 80, and 160 mg/mL solution) in methanol, and 1 mL of DPPH solution in methanol were added to each tube. The solutions were then incubated in the dark at 25°C for 45 minutes. After the designated amount of time, the absorbance of the samples was measured at a wavelength of 517 nm. The absorbance values of the samples and the blank were calculated using the following formula: Divide the difference between A blank and A extract by 100 to obtain the percentage inhibition. The percentage of inhibition is equal to the percentage of DPPH scavenging effect. absorbance for a blank in negative control. Sample absorbance is referred to as an extract. The findings were displayed as IC50 values to compare the scavenging effects of the extracts.[12]

Superoxide dismutase

After adding 1 ml of Crocus sativus alcoholic extract, 0.25 ml of ethanol, and 1.25 ml of chloroform, the mixture was centrifuged after being shaken for 15 minutes in a mechanical shaker. 1.5 ml of distilled water, 0.5 ml of pyrogallol, and 2.0 ml of 0.1 M Tris-HCl buffer pH 8.2 were added to 0.5 ml of the supernatant. A UV-VIS spectrophotometer was used to measure the change in optical density at 420 nm at 0, 1, and 3 minutes. The same procedure was used to treat control tubes with 0.5 ml of distilled water in comparison to a buffer blank. Unit/mg protein is the unit used to express the enzyme activity. The quantity of enzyme needed to achieve around 50% inhibition of pyrogallol auto-oxidation is equal to one enzyme unit.

Catalase

After adding 1.0 ml of buffer, 0.5 ml of hydrogen peroxide, and 1 ml of Crocus sativus alcoholic extract, the time was recorded. Four to twenty moles of hydrogen peroxide were used in the reaction, and they were all handled similarly. For ten minutes, the tubes were heated in a boiling water bath. A UV-VIS Spectrophotometry was used to measure the green colour that formed at 570 nm. The unit of measurement for catalase activity is µ moles of H2O2 consumed/min/mg protein.[13]

RESULTS AND DISCUSSION

Table 1: Physicochemical analysis result.[14]

S. No	Tests	Value	
		Sample	*API Reference Value*
1	Loss on drying (%)	10.65	Not more than 14%
2	Aqueous Extractive Value (%)	12.23	----------
3	Alcoholic Extractive Value(%)	9.98	----------
4	Total Ash (%)	6.9	Not more than 7.5%
5	Acid Insoluble Ash (%)	0.92	Not more than 1.0%
6	Water Soluble Ash (%)	4.57	----------

Table 2: Analysis of Primary and Secondary Metabolite.

Name of Test	AqueousExtract	Ethanol Extract
Carbohydrate		
olish test	+ ve	- ve
Benedict test	- ve	+ ve
Fehling test	+ ve	- ve
Alkaloids		
Dragendorff test	+ ve	+ ve
Wagner's test	- ve	+ ve
Hager's test	+ ve	- ve
Amino acids		
Ninhydrine	+ ve	+ ve
Protein		
Biuret test	+ ve	+ ve
Xenthoprotic test	+ ve	
Millon test	- ve	+ ve
Saponin		
Foam test	+ ve	- ve
Glycosides		
Borntrager's test	+ ve	- ve
Phenolic compound		
Phenolic test	- ve	+ ve
Steroids		
Salkowaski	- v	+ ve
Tannins		
Lead acetate	- ve	+ ve
Pot. Dichromate	+ ve	- ve

Table 3: Mean of total phenol and flavonoid content.

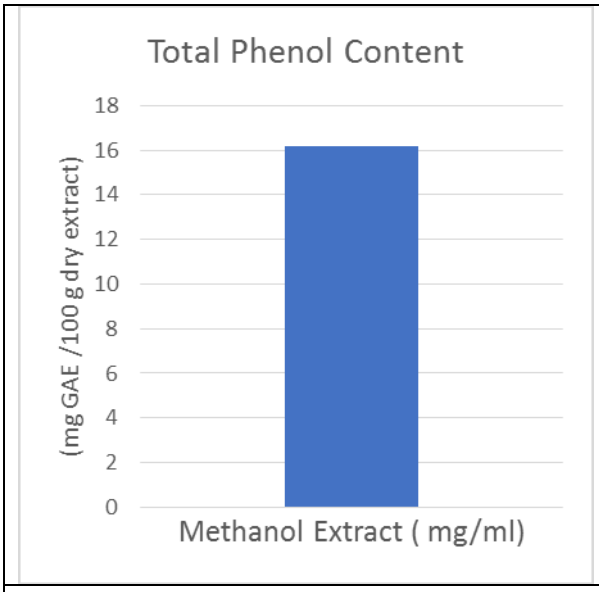
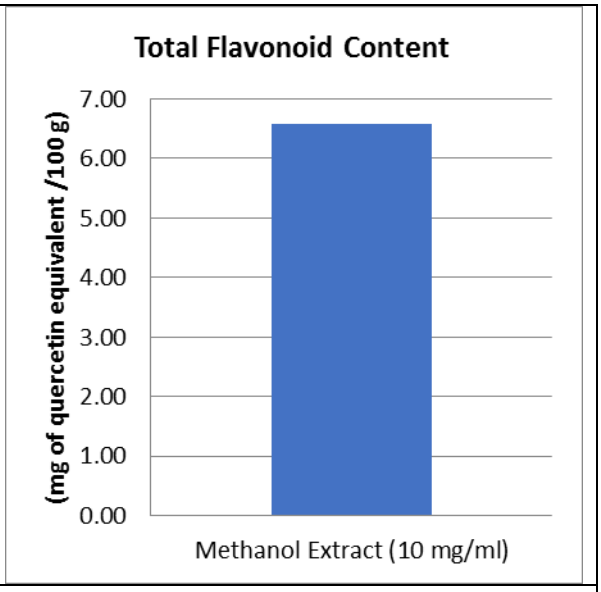
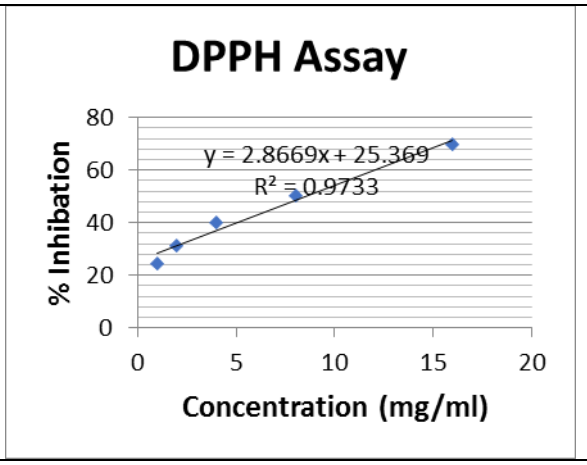
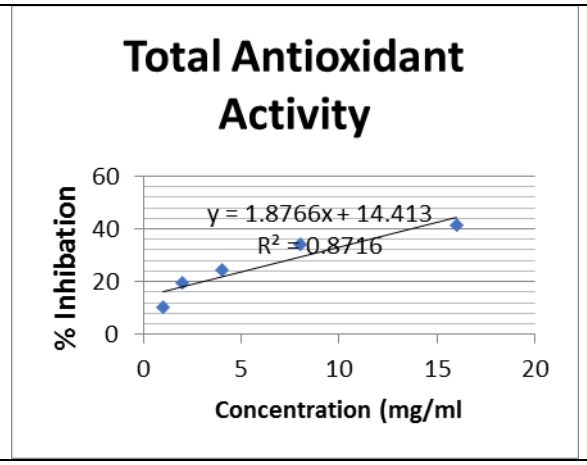
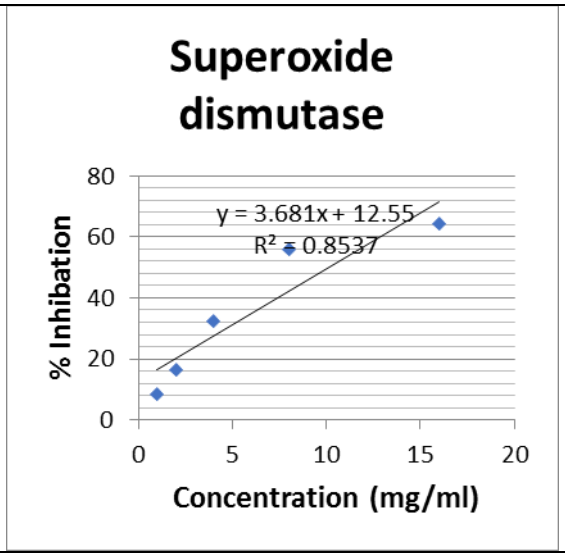
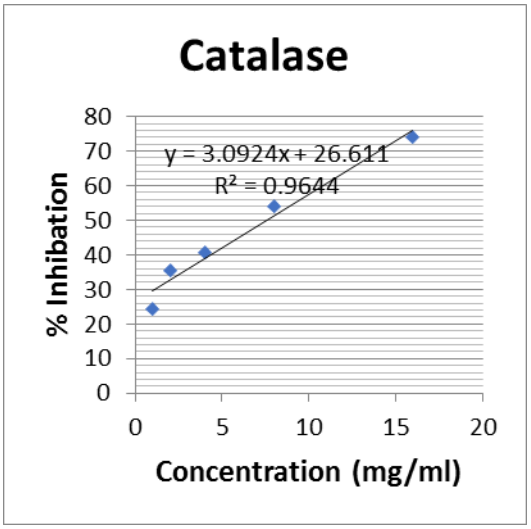
	
Total Phenol Content (mg GAE /100 g dry extract)	**Total Flavonoid Content (mg of quercetin equivalent /100 g dry extract)**
16.18±0.573	6.59±0.91

Table 4: scattered graph showing beer lambert law for DPPH, total antioxidant activity, SOD and catalase.

	
DPPH Assay (IC50 Value)	**Total Antioxidant Activity (IC50 Value)**
8.597 mg/ml	18.97122 mg/ml
Figure:1	**Figure:2**

	
Superoxide dismutase (IC50 Value)	**Catalase (IC50 Value)**
10.17387 mg/ml	7.564 mg/ml
Figure:3	**Figure:4**

DISCUSSION

total phenol and flavonoid content are the secondary metabolite which are responsible for antioxidant activity of the plant sample. Phenolics possess a wide spectrum of biochemical activities such as antioxidant, antimutagenic, anticarcinogenic as well as ability to modify the gene expression.[15] In hour sample total phenolic content is 16.18±0.573 and the total flavonoid content is 6.59±0.91 which indicates that *Crocus sativus* possess the potentially effective antioxidant characteristics. There is a positive correlation between phenolic content, flavonoid content and free-radical scavenging activity (table: 3).

The DPPH assay is used to predict antioxidant activities (table:4, figure:1) by mechanism in which antioxidants act to inhibit lipid oxidation, so scavenging of DPPH radical and therefore determinate free radical scavenging capacity of the plant sample. DPPH assay measures how antioxidants scavenge DPPH radicals, which causes the DPPH methanol solution to decolorize.[16] In this study DPPH investigation value is 8.597 mg/ml which indicates the linear relation relationship between the concentration and the absorbance of the sample solution.

Total antioxidant capacity (TAC) (table:4, figure:2) is a measure of the antioxidant activity of a sample, and is used as a diagnostic tool to investigate oxidative stress as our sample have rich phenol and flavonoid content and investigated total antioxidant activity is 18.97122 mg/ml which indicates the potential support for cell growth.

Superoxide dismutase (SOD) (table:4, figure:3) is an enzyme that protects cells from damage by converting superoxide into hydrogen peroxide and oxygen. SOD is a vital antioxidant that acts as the body's first line of defence against oxygen free radicals. It's present in almost all aerobic organisms, including plants and humans.[17] In this study investigated value of SOD is 10.17387 mg/ml which is quite potent SOD enzymatic activity in our sample types. SOD activity is determined by measuring the decrease in superoxide anions.

Catalase activity (table:4, figure:4) is the process by which the enzyme catalase breaks down hydrogen peroxide into water and oxygen.[18] The calibration curve plotted using the defined unit of catalase activity is shown in fig. A linear regression on the pooled data yielded the best linear fit over a range of 20–200 units (U) of catalase activity ($y = 3.0294x = 26.611$, $r^2 = 0.9644$).

CONCLUSION

In this study it is concluded that our plant sample (crocus sativus) is proved quality assured after the quality control analysis. All the test undertaken according to the Ayurveda Pharmacopeia of India and the obtained results were within the reference limit. The plant sample possess secondary metabolites which is helpful in plant growth. The in vitro antioxidant capacity investigation emphasizing its significance in ensuring its potential in terms of scavenging free radicals. The IC50 values for various antioxidant assays (DPPH, total antioxidant activity, superoxide dismutase, and catalase) demonstrate the extract's ability to neutralize different types of free radicals and reactive oxygen species. Lower IC50 values indicate higher antioxidant activity.

Conflict of interest

There is no conflict of interest in this study.

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