

Article

Antioxidant, antidiabetic and structural analysis of *Spinacia oleracea* leaf

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Abstract: Medicinal plants have been conventionally used to sustain health and for the treatment of many diseases such as diabetes. *Spinacia oleracea* L., common name palak, belong to the class Amaranths and family Amaranthaceous. It is used as an anti-bacterial, antidiabetic, anticancer, anti-inflammatory, antioxidant, and hepatoprotective agent. In this study, methanol, ethanol, *n*-hexane and aqueous extracts of the *S. oleracea* leaf were used to evaluate antioxidant contents, antioxidant activity, antidiabetic activity (inhibition of glycation, alpha amylase, alpha glucosidase, acetylcholinesterase). Additionally, identification of bioactive compounds and functional groups was done by Fourier transform infrared spectroscopy (FTIR) and high performance liquid chromatography (HPLC). Among all the extracts, total phenol and flavonoid contents ranged from 32.09 ± 0.99 g GAE/100 g to 78.38 ± 1.15 g GAE/100 g and 22.77 ± 0.16 g CE/100 g to 54.56 ± 0.87 g CE/100 g respectively. While DPPH reducing activity range was 45.14% -75.77%. Methanol extract was most potent as it extracted maximum antioxidant contents. Glycation and alpha amylase inhibition percentages were 15.31 to 34.28 and 19.83-36.32 among all tested samples. Whereas, 12.67% - 43.77% and 17.3% -28.04% inhibitions of alpha glucosidase and acetylcholinesterase activities were observed respectively. HPLC analysis identified various flavonoids and phenolics acids such as quercetin, gallic acid, caffeic acid, sinopic acid, cinnamic acid. FTIR revealed the presence of several phenols, amines, alkaloids, alcohols and fluoro compounds in the methanolic leaves extract. This research indicated that *S. oleracea* has wide potential to be further investigated in the future and it can prove as a wonderful natural drug for healthcare systems in the upcoming years.

Keywords: *Spinacia oleracea*; diabetes mellitus; antioxidant activity; alpha amylase.

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1. Introduction

Spinacia oleracea L. (Amaranthaceous), also known as Spinach is a dioecious green leafy vegetable of nutritional and commercial benefits. It is commonly found in Asian and European countries and used as food in processed or raw forms. This plant is a spice with around 30-60 cm height and is used to cure multiple health issues due to its numerous bioactive properties. It is acknowledged as the most powerful and perfect food due to its numerous vitamins and micronutrients. Vitamin C, vitamin A, and vitamin E are present in considerably higher concentrations and some other valuable minerals like magnesium, iron, folic acid and manganese. A high concentration of pigments like carotenoids, beta-carotene and lutein are also present in Spinach [1]. *S. oleracea* is helpful to cure oncogenesis. The therapeutic effects of Spinach, such as its enzyme inhibitory, anticancer, antioxidant, anti-inflammatory, and hypoglycemic properties, have been studied in humans and animal models [2, 3]. *S. oleracea* acts as a major remedial source for diseases such as diabetes, joint pain, asthma, cardiovascular sicknesses, urinary diseases, malignant growth, Alzheimer's infections, lung inflammation, acquired immune deficiency syndrome and optical manifestations [4]. Recently, it was studied by Gutierrez and Velazquez, [5] that Spinach can act as an anti-glycation agent. Production of reactive oxygen species and advanced glycation endproducts (AGEs) lead to diabetic

complications. Flavonoids found in *Spinacia oleracea* decline the rate of AGEs synthesis. *S. oleracea* consumption is especially useful in cardiovascular problems. It has been regarded as undoubtedly one of the best remedies nature has created for human beings. *S. oleracea* has been used as a flavoring in the whole world to improve the standard of foods, as it has a substantial nutrient value. The antidiabetic properties of *S. oleracea* leaf are attributed to steroid saponins, alkaloids, and fiber contents present in it [6]. The worldwide incidence of diabetes mellitus is increasing at an alarming rate. Poor management and fewer treatment options with more side effects have aggravated the problem. In this context, natural products derived from plants are recommended as a safer substitute and are being used by humans since ancient times [7]. Diabetes and hypertension disorders are treated by many herbal remedies and maintain the health of individuals around the whole world. Herbal medicines are used to lower blood glucose levels. In medicinal plants, spinach leaves play a vital role in managing type 2 diabetes through nutritional therapies [8]. Although different parts of *S. oleracea* have been extensively studied for diverse biological activities, however, no comparative analysis of plant leaves in different extracts/solvent fractions (aqueous, methanol, ethanol, *n*-hexane) is available to-date. The current study evaluated phenolic contents, flavonoid contents, and antioxidant and anti-diabetic (glycation and enzymes inhibition) activities of *S. oleracea* leaf extracts and fractions. Structural characterization and functional group identification were made by high-performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (FTIR) techniques.

2. Materials and Methods

S. oleracea (Spinach) fresh leaves were randomly collected from the local vegetable market of Faisalabad. After authentication by the Department of Botany, University of Agriculture, Faisalabad, leaves were washed, dried and ground into powder. As described earlier, aqueous, methanol, ethanol and *n*-hexane extracts/fractions were prepared [9]. Extracts and Folin-Ciocalteu reagent (10%) were mixed. After adding 3 mL of 1% Na₂CO₃, it was incubated for 120 minutes at ambient temperature. TP as gallic acid equivalent (g gallic acid/100 g dry weight) was calculated by taking absorbance at 760 nm. For the estimation of TF; extracts, 10% AlCl₃, distilled water and potassium acetate (1 M) were mixed. TF as g catechin equivalents/100g dry weight) was noted by taking absorbance at 510 nm [9]. Extracts were mixed with 5 mL DPPH and incubated for 30 minutes at room temperature. Absorbance was taken at 517 nm. Inhibition percentage was calculated: Radical Scavenging (IC₅₀%) = 100 × (Absorbance blank – Absorbance test sample / Absorbance blank). BHT (Butylated hydroxy toluene) was the standard control [10]. A reaction mixture of 100 mg D-glucose and 10 mg bovine serum albumin (BSA) prepared in 67 mM sodium phosphate buffer (pH 7.2) was incubated at 37 °C for 2 days with the sample. The absorbance of 0.2 mL diluted reaction solution was measured at 370 nm (excitation wavelength) and at 440 nm (emission wavelength) by ELISA reader (BioTek, Winooski, VT, USA). As a control, a solution without D-glucose was utilized. The chemical metformin was employed as a reference compound [Hussain et al., 2021]. % inhibition = [Abs 440/Abs 370- Abs 440] × 100. An assay of alpha amylase inhibition was performed according to [9]. Porcine pancreatic α-amylase (EC 3.2.1.1, Sigma Chemical Co), extracts, SF (500 μL) and 500 μL amylase solution (0.5 mg/mL) in buffer (0.02 M; pH 6.9) were incubated at 25° C. After 10 minutes, 500 μL starch solution (1%) was added at fixed time intervals and incubated. The reaction was blocked by adding 1 mL of dinitrosalicylic acid (DNS) reagent, heated in a boiling water bath for 5 minutes, cooled to room temperature and diluted with 10 mL distilled water. Absorbance was noted against blank at 540 nm. % inhibition was calculated as: ([Ac- As/Ac]) × 100. Ac is the absorbance of the control, and As is the absorbance

of test samples. For control (negative), the buffer was used, and positive control was acarbose drug. Acetylcholinesterase (AChE) inhibition assay was conducted by incubating the mixture of phosphate buffer of pH 8, test samples, AChE (Aldrich Germany) and DTNB (5,5-dithio-bis-2-nitrobenzoic acid) solution at 25°C. The enzyme used was *Electrophorus electricus* (electric eel). Negative control was reaction mixture without inhibitors, and physostigmine was a positive control. After the addition of substrate acetylcholine iodide, absorbance (412 nm) was measured. % Inhibition: $(\text{Abs Cntrl} - \text{Abs smpl} / \text{Abs Cntrl}) \times 100$ [11]. Alpha-glucosidase enzyme (source: *S. cerevisiae*, Sigma Aldrich, USA) solution (500 μL) and 100 μL plant extracts were mixed and incubated for ten minutes at 25°C. About 500 μL of substrate solution (*p*-nitrophenyl- α -D-glucopyranoside) was added then again kept at room temperature for five minutes. The absorbance was taken at 405 nm. Extraction solvent was used to replace the extract in the negative control sample, whereas acarbose was used as a positive control. % Inhibition: $(\text{Abs Cntrl} - \text{Abs smpl} / \text{Abs Cntrl}) \times 100$ [12]. The ChromNAV 2.0 software, a powerful data system was used. Spinach samples were dried and hydrolyzed. This hydrolyzed mixture was taken in 0.5g quantity and mixed with 20 mL methanol. Then 10 ml of 1M solution of hydrochloric acid was added to this reaction mixture. This mixture was subjected to a gentle shake, and then sonification was done for 15 minutes, the mixture was placed on a water bath at 90 °C for 2 hours. The resulting mixture (20 μL) was added to the HPLC column. Chromatographic conditions were solvent A (H₂O: Acetic acid-94:6, pH=2.27) and solvent B (Acetonitrile CAN 100%) as mobile phase, stationary phase (Shim-pack CLC ODS(C-18), 25cm \times 4.6mm, 5 μm) with 1mL min⁻¹ rate of flow. Reading was taken at 280nm. Different compounds were identified according to their retention time [13]. FTIR spectra were recorded with an FTIR instrument (IFS 25 model, Germany). OPUS software was used for the measurement, processing and evaluation of IR spectra. Powdered leaf sample and potassium bromide were mixed, and by applying pressure, a thin layered film was prepared. The data were matched with reference to detect the functional groups [14]. All the results were indicated as means \pm SD (standard deviation) or percentage. Analysis of variance (ANOVA) was used to analyze Microsoft Excel 360 at a level of significance $p < 0.05$.

3. Results

3.1 TPC, TFC and Antioxidant activity

Results of antioxidant contents and activity are presented in table 1. TPC was in the range of 32.09 ± 0.99 g GAE/100 g to 78.38 ± 1.15 g GAE/100 g. Methanol extract showed maximum TPC levels. In other fractions, TPC contents in descending order were as: aqueous > *n*-hexane > ethyl acetate. TFC of spinach leaf varied from 22.77 ± 0.16 g CE/100 g to 54.56 ± 0.87 g CE/100 g. The methanol sample was most potent as it extracted optimal flavonoids. TFC contents in descending order are as: *n*-hexane > ethyl acetate > aqueous. Regarding antioxidant activity, highly significant DPPH reducing activity was shown by methanol extract (75.77%). Antioxidant activities of other extracts and fractions were in the range of 45.14% - 75.77%, and the tendencies in ascending order are as follows: ethyl acetate < aqueous < *n*-hexane.

3.2 Antidiabetic activities

Prominent glycation inhibition (15.31 to 34.28) was observed in the present study. Tendency in declining direction was as methanol>ethanol>*n*-hexane>aqueous. In the current research, alpha-amylase and alpha-glucosidase inhibitions (percentage) ranged from 19.83-36.32 and 12.67-43.77, respectively. All extracts showed anti-acetylcholinesterase activities in the range of 17.3% to 28.04% (Fig.1). Trends in ascending order were as: aqueous<*n*-hexane<ethanol<methanol.

Table 1. Antioxidant contents, antioxidant and antiglycation activities

Extract/ fractions	TPC	TFC	Antioxidant activity	Antiglycation activity
ME	78.38 ± 1.15*	54.56 ± 0.87*	75.77*	34.28*
EE	70.22 ± 1.27	28.62 ± 0.19	45.14	27.12
NHE	63.18 ± 0.58	22.77 ± 0.16	63.43	16.98
AE	32.09 ± 0.99	39.25 ± 0.25	55.18	15.31
Control	-	-	91.56	56.25

Data is represented as mean ± SE or percentage (n=3). In terms of g GAE/100 g for TPC (total phenolic contents) and g CE/100 g for TFC (total flavonoid contents) and antioxidant activity represented as a percentage. * Significant at p < 0.05. ME: methanol extract, EE: ethanol extract, NHE: n-hexane extract, AE: aqueous extract. Positive controls: BHT; Butylated hydroxytoluene (antioxidant activity), metformin (antiglycation assay)

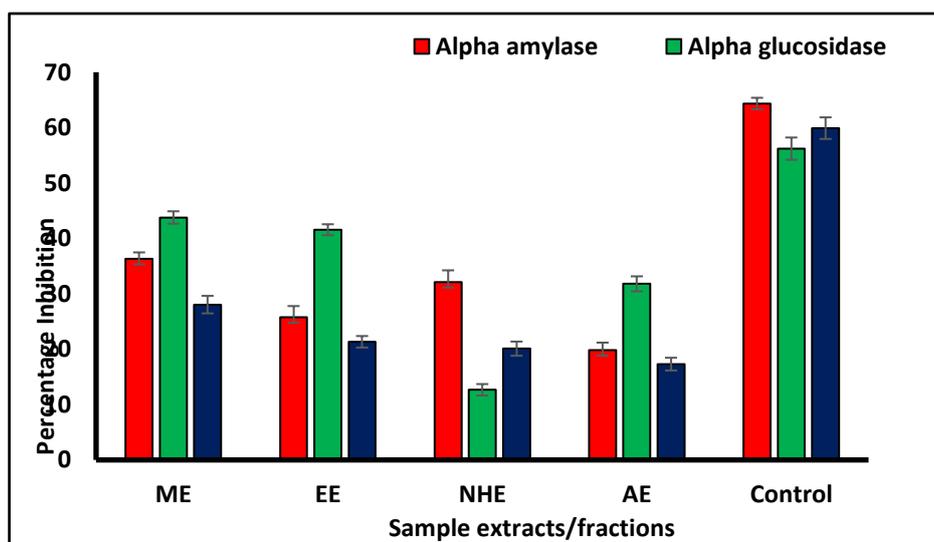


Figure 1. Enzyme inhibition assay

Data is represented as mean ± SE (n=3). ME: methanol extract, EE: ethanol extract, NHE: n-hexane extract, AE: aqueous extract. Positive controls: acarbose (alpha-amylase inhibitory assay), physostigmine (acetylcholinesterase inhibitory assay), acarbose (alpha-glucosidase)

3.3 Structural characterization

3.3.1 HPLC analysis

Bioactive compounds that HPLC detected in the methanolic leaves extract of *S.oleracea* are given in table 2. Compounds identified by HPLC are quercetin, gallic acid, caffeic acid, p-coumaric, benzoic acid, m-coumaric acid, cinnamic acid and sinopic acid. Quantities and concentrations calculated by HPLC for quercetin, gallic acid, caffeic acid, p-coumaric, benzoic acid, m-coumaric acid, cinnamic acid and sinopic acid are 44.37 ppm, 12.35 ppm, 12.61 ppm 2.68 ppm, 39.76 ppm, 8.43 ppm, 6.71 ppm and 2.51 ppm respectively. Out of all these compounds, only quercetin is flavonoid, while all others are phenolic compounds.

Table 2: Quantification of different flavonoids and phenolic compounds

Retention Time	Area (mv. s)	Area %	Amount (ppm)	Compound Name	Nature of compound
2.813	2770.535	12.2	44.37	Quercetin	Flavonoid
4.565	343.901	1.5	12.35	Gallic acid	Phenolic
12.787	274.707	1.2	12.61	Caffeic acid	Phenolic
17.933	648.471	2.9	2.68	p-Coumaric	Phenolic
14.827	371.762	1.6	39.76	Benzoic acid	Phenolic
20.400	411.050	1.8	8.43	M-coumaric acid	Phenolic
25.173	191.798	0.8	6.71	Cinnamic acid	Phenolic
26.127	191.773	0.8	2.51	Sinopic acid	Phenolic

The chromatogram of HPLC shown in figure 2 indicates different quantities and concentrations of different compounds, and the determiner in this chromatogram is the compound's retention time inside the mobile phase or HPLC. This chromatogram shows only one flavonoid present in the leaves of Spinach which is quercetin, detected at 2.813 retention time corresponding to the 44.37 ppm concentration. Whereas the phenolic compounds found by HPLC in the methanolic leaves extract of Spinach are gallic acid, benzoic acid, caffeic acid, p-coumaric acid, benzoic acid, m-coumaric acid, cinnamic acid, sinopic acid, having a different concentration in the Spinach.

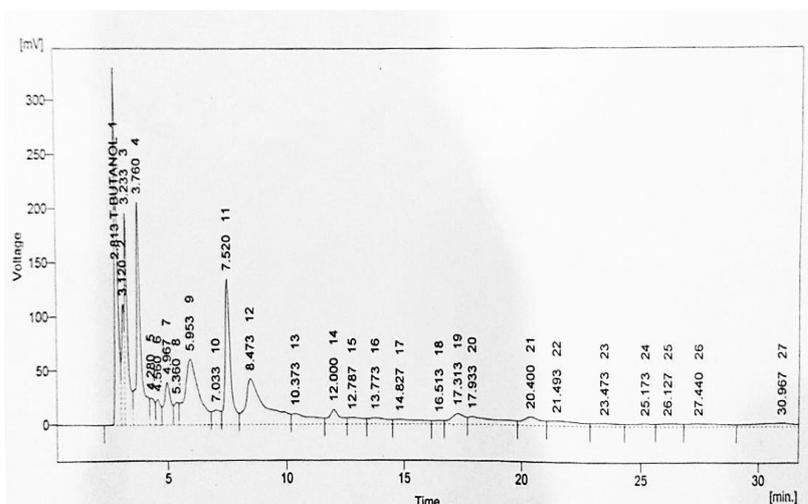


Figure 2. HPLC profile of *S.oleracea* leaves extract

3.3.2 FTIR analysis

This Interferogram generated by FTIR shown in figure 3 represents compounds identified in the spinach leaf extract prepared in methanol. A strong peak at 3600 cm^{-1} indicated the presence of alcohols. A band obtained at 3000 cm^{-1} indicated the presence of amine salts in the sample. The band at 2840 cm^{-1} correspond to the presence of alkanes. Two medium bands at 2550 cm^{-1} and 1765 cm^{-1} identified the presence of carboxylic acid.

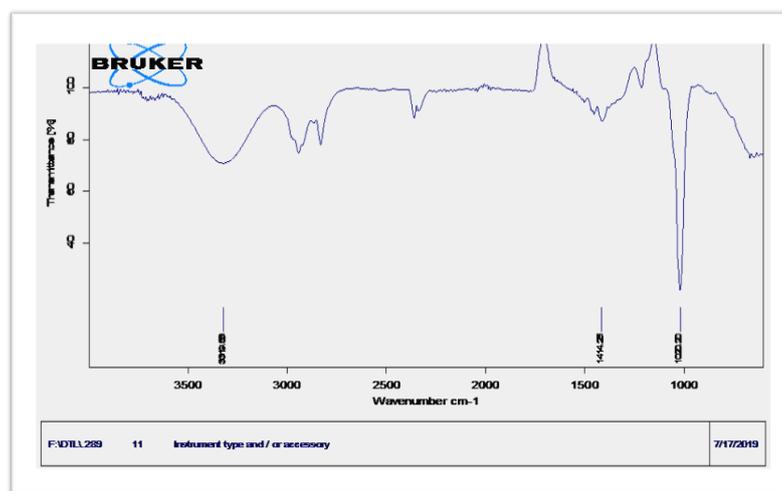


Figure 3. FTIR spectra of methanolic extract of *S.oleracea*

Table 3: FTIR spectrum of *S.oleracea* methanolic leaves extract

Peak no	Characteristic absorption	Identified Functional Groups	Compounds Class
1	3600	O-H and N-H stretching	Alcohol, Amines
2	3000	N-H stretching	Amine Salt
3	2840	C-H stretching	Alkane
4	2550	O-H stretching	Carboxylic acid
5	1760	C=O stretching	Carboxylic acid
6	1500	C=C stretching	Alkene
7	1430	O-H stretching	Carboxylic
8	1265	C-F stretching	Flouro
9	1120	C-N stretching	Amine
10	1060	C-N stretching	Amine
11	1010	S-O stretching	Sulfoxide

Table 3 shows the values of absorption as predicted by the FTIR identified different functional groups present in the methanolic leaves extract of *S.oleracea*. A strong peak at 3600 cm^{-1} indicated the presence of alcohols. A band obtained at 3000 cm^{-1} indicated the presence of amine salts in the sample. The band at 2840 cm^{-1} corresponds

to the presence of alkanes. Two medium bands at 2550 cm^{-1} and 1765 cm^{-1} identified the presence of carboxylic acid.

4. Discussion

4.1 TPC, TFC and Antioxidant activity

Determination of antioxidant constituents like phenols and flavonoids in natural products provides a link to their medicinal attributes. These bioactive can hunt the reactive species or free radicals and have the capacity to convert them into non-toxic components. Oxidants are the causative agents for a variety of diseases such as diabetes mellitus, arthritis, cardiovascular ailments, cancer, Alzheimer's infections, acquired immunodeficiency syndrome etc. Natural antioxidant producers include fruits and vegetables. Various colors, types and tastes are present in vegetables, and their inherent nutrient composition demands their use in daily food. Each vegetable is given preference depending upon its seasonal availability, dietary habits, nutrient composition and medicinal properties [15]. Phytochemicals in green leafy vegetables like Spinach protect the cells from oxidative damage and control the function and expression of some genes involved in metabolism, immunity, and redox defenses [16]. In the current study, *S.oleracea* leaf extracts and fractions were investigated for total phenolics, flavonoids contents, and their antioxidant activity measured as DPPH reducing potential. However, most of the tested samples exhibited TPC and TFC, their concentrations in fractioned samples depending upon solvent's ability to extract phytoconstituents. Current results are in accordance with the recent findings. Dasgupta and Patel [6] observed 60-107 mg GAE/g TPC and 25-114 mg QE/g TFC in different extracts of *S.oleracea* leaf. Previously, Banerjee et al. [17] reported 18.70 mg/g TPC and 14.81 mg/g TFC in methanolic extracts of spinach leaf. In another study, Gunathilake and Ranaweera [18] measured the total phenolic content of 3.81 mg GAE/g of dry weight, contrary to the current study findings. Bergquist et al. [19] calculated the total flavonoid content present in Spinach to be 13-23 mg per gram of dry weight. These results are in agreement with current results. Furthermore, Finten et al. [20] demonstrated that phenolic concentration and antioxidant activity could be increased when Spinach is grown in intense light. Excessive synthesis of oxidative chemicals can lead to numerous pathological conditions. Ingestion of fruits and vegetables is inversely related to oxidative stress. Phytoconstituents not only scavenge these reactive species but also have the anti-inflammatory, anticancer, anti-ageing and preventive potential for neurodegenerative diseases, diabetes mellitus, and heart diseases. Significant antioxidant activity was observed in methanol extract. Banerjee et al. [17] reported $455.13 \pm 2.05\text{ }\mu\text{g/mL}$ in methanol extracts of *S. oleracea* leaves. Similar to current inferences, Dasgupta and Patel [6] documented 42.33% - 96.33% antioxidant activity of *S.oleracea* leaf in different extracts.

4.2 Antidiabetic activities

The glycation process takes place under normal and pathological conditions. It changes both the structure and function of proteins, lipids and nucleic acids. This process is extracellular as well as intracellular. Firstly, the formation of non-enzymatic binding of glucose with amino groups occurs. Later, through a series of rearrangements, advanced glycation end products are formed. Altered proteins can exhibit altered function and lead to numerous diseases [21]. Inhibiting alpha-amylase and alpha-glucosidase activities will block the conversion of polysaccharides into glucose and manage hyperglycemia, thereby reducing intestinal glucose absorption. Although different synthetic blockers are available, yet several side effects minimize consumer reliability. As an alternate, phytoconstituents can restrict enzyme activity but up to a certain variable limit [22]. Prominent glycation inhibition was observed in the present study. Contrary to current findings, Ishioka et al., [23] reported minimal polyphenols and antiglycation activity of *S.oleracea* leaf extracts.

Although, Gutierrez and Velazquez, [5] indicated flavonoids that may block the glycation process. The difference in the results may be due to differences in solvents used, experimental setup and measurement units. Inhibiting alpha-amylase and alpha-glucosidase activities will block the conversion of polysaccharides into glucose and manage hyperglycemia, thereby reducing intestinal glucose absorption. Although different synthetic blockers are available, yet several side effects minimize consumer reliability. As an alternate, phytoconstituents can restrict enzyme activity but up to a specific variable limit [22]. The latest scientific comprehensions suggest that ingestion of fresh plants defend the body against enduring critical diseases. Dark green leafy vegetables are recognized to amend pathology. In the current research, alpha-amylase and alpha-glucosidase inhibitions (percentage) were observed. Synthetic drugs were more potent inhibitors in the case of both enzymes. However, Barkat et al. [24] detected 20-80 percent inhibitions of alpha-amylase by Spinach at different growth levels and stated that maximum inhibition would be demonstrated by mature Spinach. Nutrition-based treatment and management of neurodegenerative conditions are required for proper brain activity. Foods rich in vitamins, minerals, secondary metabolites and antioxidants such as *S.oleracea* (Family: *Amaranthaceae*) can be an active neuro-nutrition. According to Jiraungkoorskul [25], Spinach may act as an anti-Alzheimer's plant. All extracts showed anti-acetylcholinesterase activities in the current study. Yadav et al. [26] observed ameliorating effects of *S.oleracea* seed extracts in experimental animals. Treatment with Spinach reduced acetylcholinesterase activity and improved neuropsychiatric symptoms.

4.3 Structural characterization

4.3.1 HPLC analysis

Similar to current finding, Bergman et al., [27] identified gallic acid, chlorogenic acid, protocatechuic acid, alpha-resorcylic acid, vanillic acid, syringic acid, p-hydroxybenzoic acid, m-hydroxybenzoic acid, gentisic acid, p-coumaric acid, m-coumaric acid, ferulic acid, o-coumaric acid, and sinapic acid in a spinach leaf. Murcia et al. [28] reported similar compounds present in the leaves of Spinach, such as quercetin, spinacetin, ferulic acid, and caffeic acid. He also suggested that the levels of these compounds are higher in summer as compared to winter. Polyphenols are the compounds responsible for the protection of cells from biological oxidative stress and improving their life. The difference between the current and past studies is different, possibly due to the difference in the solvent used and the identification methods used.

4.3.2 FTIR analysis

Fourier transform infrared spectroscopy is the most suitable and speedy method for identification and determination of concentrations of different chemical and functional groups present in the sample under consideration, and we can easily conclude based on this information what kind of compounds and molecules are present in the substance; thus we can establish a reliable structure of substance present in the sample. Thus FTIR is used to identify various useful substances like acids, phenols, alkalies, alkyl groups, carboxylic and fluoro compounds present in the sample. FTIR showed that *S. oleracea* contains several phenols, amines, alkaloids, alcohols, and fluoro compounds in methanolic leaf extract. The plant under observation has remarkable effects as antimicrobial, antioxidant and antidiabetic drug against bacterial strains, cellular oxidative stress and blood glucose levels. Recently, Younis et al., [29] detected functional groups ester, amine, ketone, aldehydes (bonds: N-H, O-H, C-H, C = O, C = C, C = N, C = N) in spinach leaf by FTIR technique.

5. Conclusions

This research indicated that *S. oleracea* has vast potential to be further investigated in the future and it can prove as a wonder drug for healthcare systems in the upcoming years

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