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Research article

Antioxidant, and enzyme inhibition effects of chia (*Salvia hispanica*) seed oil: A comprehensive phytochemical screening using LC-HR/MS ☆

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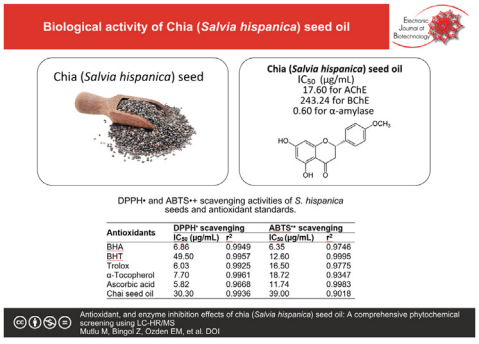
GRAPHICAL ABSTRACT



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ABSTRACT

Background: In this study, the antioxidant and anti-Alzheimer's disease properties of Chia (*Salvia hispanica*) seed oil (CSO) were determined for the first time. Three different metal reduction and two different radical scavenging methods were used to determine the antioxidant properties of CSO. It exhibited higher antioxidant activity than vitamins E and C in the CUPRAC method.
Results: CSO has shown excellent efficacy in the antioxidant methods used in this study. In the DPPH radical scavenging method, CSO exhibited higher radical scavenging potential than BHT, a standard and synthetic antioxidant. The anti-Alzheimer's disease properties of CSO were determined by inhibition of acetylcholinesterase enzyme and its IC_{50} value (17.60 μg/mL) was found to be close to the IC_{50} value

Abbreviations: ABTS, 2,2́-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); AChE, Acetylcholinesterase; AD, Alzheimer's disease; AZA, acetazolamide; BChE, butyryl-cholinesterase; BHA, butylated hydroxyanisole; CSO, Chia (*Salvia hispanica*) seed oil; CUPRAC, Cupric ions reducing power; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); ESI, electrospray ionization; FRAP, ferric reducing antioxidant power; GC/MS, Gas chromatography/Mass chromatography; GC-FID, Gas chromatography-Flame ionization detector; hCA II, human carbonic anhydrase II isoenzymes; HPLC, High-performance liquid chromatography; IC_{50}, half maximal inhibitory concentration; LC-HRMS, Liquid chromatography–high-resolution mass spectrometry; LC-HRMS, Liquid chromatography-high-resolution mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; ROS, reactive oxygen species; TCA, Three chloroacetic acid; TPTZ, tripyridyltriazine.

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of tacrine (8.82 μg/mL), the standard inhibitor of the enzyme. Inhibition properties of α-glycosidase enzyme and human carbonic anhydrase II isoenzymes were also studied. It was understood that CSO inhibited both enzymes at a lower rate than standard inhibitors. Also, the total phenolic and flavonoid contents of CSO were determined as 784.44 μg gallic acid equivalent (GAE)/mL oil and quercetin 150.00 μg QE)/mL oil, respectively. In addition, LC-HRMS chromatography application was performed to understand the phenolic content of CSO. It was determined that isosakuranetin (29.07 mg/L oil) was the most abundant polyphenolic compound in CSA. Also, seven polyphenolics of the studied remained below the detectable amount.
Conclusions: It was found that CSO had effective antioxidant activity, polyphenolic contents and potent enzyme inhibition properties, associated with some global disease.
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1. Introduction

Plants are integral to human health in multiple ways. Their medicinal properties, nutritional value, air-purifying abilities, and contributions to biodiversity all make them vital for our well-being [1]. The use of plants for medicinal purposes, often referred to as herbal medicine or herbalism, has a long history dating back thousands of years. Plants have been a fundamental source of healing for various cultures around the world, and their medicinal properties continue to be relevant today [2,3,4]. Herbal medicine has been practiced for centuries in different cultures, including Traditional Chinese Medicine, Ayurveda, Native American healing practices, and more. This historical knowledge and experience have contributed to a vast repository of information about plant-based remedies and their effects on health [5]. Plants contain bioactive compounds such as flavonoids, alkaloids, terpenes, and polyphenols that have demonstrated therapeutic properties. These compounds are called secondary metabolites and may have antioxidant, anti-inflammatory, antimicrobial, analgesic, and other beneficial effects on the body. Secondary metabolites are organic compounds produced by plants that are not directly involved in their primary metabolic processes such as growth, development, and reproduction. Unlike primary metabolites like carbohydrates, proteins, and fats, which are essential for basic life functions, secondary metabolites are often considered to have non-essential roles in the plant's survival. However, they play crucial roles in various ecological interactions, defense mechanisms, and human applications. Many secondary metabolites from plants have many medicinal properties and are used in traditional and modern medicine [6,7]. Some secondary metabolites serve as precursors for the synthesis of pharmaceutical drugs. For example, the alkaloid morphine from the opium poppy is used to produce pain-relieving medications. Other well-known examples include aspirin (originally derived from willow bark) and the anti-malarial drug quinine (derived from the cinchona tree) [8,9,10].

The chia (*Salvia hispanica*) is a flowering plant that belongs to the mint family, *Lamiaceae*. It is native to central and southern Mexico and Guatemala and has a long history of cultivation by indigenous peoples in those regions. *S. hispanica* plants are known for their small, nutrient-rich seeds, which have gained popularity as a super food due to their numerous health benefits. Chia seed oil (CSO) are the most well-known part of the plant and are often consumed for their nutritional value [11,12]. They are small, oval-shaped seeds that can vary in color from white to dark brown or black. CSO are rich in nutrients, including omega-3 fatty acids, dietary fiber, protein, antioxidants, and various vitamins and minerals. Consuming chia seeds has been associated with various health benefits, including improved heart health by reducing blood pressure and cholesterol levels, better digestion due to their high fiber content, and potential anti-inflammatory effects. CSO can also aid in weight management by promoting a feeling of fullness [13,14,15]. In recent years, CSO have gained popularity as a health food, leading to increased cultivation and commercialization. They are now widely available in grocery stores and health food markets [16]. CSO is a type of vegetable oil that is extracted from chia seeds, the small nutrient-rich seeds of the *S. hispanica* plant. CSO is gaining popularity as a healthful oil due to its nutritional content, particularly its high concentration of fatty acids and other beneficial seconder metabolites. These fatty acids play a crucial role in heart health, brain function, and overall well-being [17,18,19]. It has been reported that the anti-inflammatory properties of the fatty acids of CSO may help alleviate the chronic pain associated with various health conditions. CSO's nutritional profile makes it suitable for skincare and haircare products. Its fatty acids and antioxidants can help moisturize the skin, reduce inflammation and promote healthy hair growth. Some cosmetic products incorporate CSO for these potential benefits [20,21,22,23].

Free radicals are highly reactive molecules that can lead to oxidative stress in the body, which is linked to various chronic diseases [24]. Reactive oxygen species (ROS) include free radicals that play an important role in oxidation reactions in metabolism. Excessive production of ROS is harmful and can cause the breakdown of cell membranes, damage to membrane proteins, DNA mutations and unwanted oxidation of many biomolecules [25]. As a result of this process, it is known that they cause many diseases such as aging, cancer, arteriosclerosis, diabetes mellitus, inflammation, liver damage, skin damage, and coronary heart diseases [26,27]. Antioxidants can inhibit the oxidation of biomolecules by breaking the chains of free radical reactions by donating their own electrons to free radicals [28]. They are found in plants and other natural sources that play an essential role in protecting cells and tissues from damage caused by harmful molecules called free radicals and ROS [29,30]. They neutralize these free radicals, helping to maintain the overall health of cells and tissues [31,32,33]. Plants are rich sources of antioxidants, and they produce a wide variety of these compounds to protect themselves from environmental stressors like UV radiation, pathogens, and other potential threats. When we consume plant-based foods, we also benefit from these antioxidants [34]. So, the consumption of bioactive antioxidant compounds from plants or their processed products like oils is linked to the prevention and decreased risk of above-mentioned degenerative diseases [35,36].

Alzheimer's disease (AD) is a neurodegenerative disorder that primarily affects memory, thinking, and behavior. One of the key

features of AD is the presence of abnormal protein aggregates in the brain, including amyloid-β plaques and tau tangles [37]. These aggregates lead to the progressive loss of neurons and synapses, which ultimately results in cognitive decline. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are enzymes that play a crucial role in the cholinergic system, which is involved in transmitting nerve signals in the brain and other parts of the nervous system. Both enzymes are responsible for breaking down the neurotransmitter acetylcholine (ACh) after it has transmitted its signal, terminated the signal and allowed the nerve cell to reset for the next transmission [38,39,40]. The cholinergic hypothesis is one of the earliest and most influential theories about the underlying causes of AD [41]. It suggests that a deficiency of ACh, brought about by the loss of cholinergic neurons and the breakdown of the cholinergic system, contributes to the cognitive deficits seen in Alzheimer's patients. This deficiency is partially due to the accelerated breakdown of ACh by AChE and BChE enzymes [42,43].

Diabetes mellitus (DM) is a metabolic disease characterized by defective insulin secretion or insulin action, which leads to hyperglycemia and disturbed carbohydrate metabolism [44,45]. The hydrolysis of carbohydrates in the diet is the main source of free blood sugar. This process is regulated by enzymes such as α-amylase and α-glucosidase. Of these, α-amylase is primarily produced in the salivary glands and the pancreas. It plays a crucial role in the digestion of polymeric carbohydrates. The enzyme's main function is to break down complex carbohydrates, such as starches and glycogen, into simpler sugar units like maltose and glucose, which can then be absorbed by the body [46]. α-Amylase breaks down dietary starch into maltose dimers with α-D-glucosidase activity. The resulting maltose is digested into glucose in the presence of the maltase enzyme. This rapid breakdown of dietary starch into glucose causes type II diabetes (T2DM), which is responsible for the increase in blood sugar levels [47]. In people with diabetes, maintaining stable blood sugar levels is important. After a meal, the rise in blood sugar levels can be particularly problematic. α-Amylase plays a role in this postprandial blood sugar spike. Normally, α-amylase breaks down carbohydrates in the digestive tract, leading to an increase in glucose levels. In people with diabetes, this process can contribute to elevated blood sugar levels [48].

Carbonic anhydrase (CA) is an enzyme that plays a crucial role in various physiological processes across different organisms [49,50,51]. Its importance stems from its involvement in facilitating the inter conversion of carbon dioxide (CO_2) and bicarbonate (HCO_3^-) ions, a reaction that is essential for maintaining acid-base balance, regulating pH, and facilitating various biochemical reactions [52,53]. CA accelerates the reaction between CO_2 and water to produce carbonic acid (H_2CO_3), which rapidly dissociates into HCO_3^- ions and protons. This reaction helps regulate the pH of bodily fluids, including blood and cellular fluids [54]. Maintaining proper pH levels is crucial for various biochemical reactions, enzyme function, and overall physiological stability [55,56]. The CA enzyme has been targeted for drug development, especially in conditions like glaucoma, epilepsy, altitude sickness, and certain cancers [57]. Inhibition of CA can have therapeutic effects in these conditions by affecting the underlying physiological processes that the enzyme is involved in [58].

Phenolic compounds are a diverse group of secondary metabolites that play a important role in the biological activities of plants. These compounds are widely distributed throughout the plant kingdom and are known for their various functions, including defense against pathogens, UV radiation protection, and communication with other organisms [59]. Phenolic compounds encompass a wide range of molecules, such as phenolic acids, flavonoids, lignans, tannins, and stilbenes, each with its own unique chemical structure and properties [60]. Studying phenolic compounds in plants is of significant importance due to their diverse range of biological activities and potential benefits for human health, agriculture, and industry [61]. Many traditional herbal medicines have utilized plant-derived phenolic compounds for their therapeutic effects. Modern research aims to identify and isolate specific phenolic compounds that could serve as the basis for developing new pharmaceuticals or nutraceuticals with targeted health benefits [62]. It is well known that the antioxidant capacity of plants or natural products obtained from plants is due to the phenolic compounds found in their structures [63,64,65]. The antioxidant capacity of phenolic compounds depends on the number and position of hydroxyl groups in the molecule. Phenolic compounds are not active antioxidants unless the substitution in the ortho- or para- position increases the electron density in the hydroxyl group (-OH) and decreases the oxygen-hydrogen bond energy [66,67,68,69]. The presence of phenol groups in the meta- position of phenolic compounds is more effective. In addition, the presence of electron-withdrawing or repulsive groups in phenolic compounds, steric and electronic effects are responsible for the antioxidant activities of phenolic antioxidants [70].

In this study, the polyphenol content of CSO and its antioxidant, anti-Alzheimer's disease, antidiabetic effects, and inhibition potential towards hCA II were investigated. In addition, the polyphenol content of CSO were determined using LC-HR/MS.

2. Materials and methods

2.1. Chemicals

2,2́-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), α-tocopherol, 2,9-dimethyl-1,10-phenanthroline (neocuproine), Trolox, butylated hydroxyanisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH·), and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ascorbic acid, fumaric acid, chlorogenic acid, caffeic acid, naringin, vanillic acid, syringic acid, rutin, rosmarinic acid, p-coumaric acid, salicylic acid, quercetin, luteolin, naringenin, chrysin, and emodin were purchased from Sigma-Aldrich. Hyperoside, luteolin 7-glycoside, orientin, (+)-trans-taxifolin, quercitrin, hispidulin, apigenin, hederagenin, and acacetin were obtained from TRC, Canada. Verbascoside and luteolin-7-rutinoside were purchased from HWI Analytik GMBH and Carbosynth Ltd., respectively. Hesperidin was purchased from J&K Co. Ltd., Isosakuranetin, dihydrokaempferol, and penduletin were purchased from Phytolab. Apigenin 7-glucoside was obtained from the EDQM CS. Myricetin was purchased from Carl Roth GmbH & Co. Nepetin and caffeic acid phenethylester were purchased from Supelco and the European Pharmacopoeia, respectively.

2.2. Preparation of chia seed oil

Chai (*S. hispanica*) seeds were imported to Türkiye from South America. Chia seeds were obtained through a process called cold pressing or cold expeller-pressing. Chia seed oil is obtained completely mechanically and without using any chemicals by cold pressing method. The device used in my cold pressing method is manufactured in Izmir province of Turkey (Tok 55, Serial number: 60–65). This pressing device is a device that can apply up to 20 tons of pressure and has a screw press feature. The pressure required in the cold pressing method is provided by a very large screw cylinder. Chia seeds are poured into this cylinder. In this way, the oil is thrown out from the bottom of the machine and discharged. The chia seed pressing process is carried out at a temperature not exceeding 40°C. Chia seed oil was first filtered through a cloth filter and then through a paper filter. Samples were taken

into 250 mL brown glass bottles without leaving any headspace and stored at +4°C until analyzed. A total of 25 g oil was obtained from 100 g seeds. This method involves mechanically extracting the oil from CSO without the use of heat or chemicals, which helps to preserve the oil's nutritional quality and flavor. Chai seeds were cleaned and dried to remove any impurities or moisture. The dried chia seeds were fed into the press, where they were gradually compressed as they moved through the barrel. As the seeds were compressed, the oil was squeezed out of them. The oil then was flowed out of the press and was collected, while the remaining CSO solids were expelled from the other end of the press. The mixture of oil and some residual solids were collected in a container. To separate the oil from any remaining solids, the mixture was subjected to a filtration process that removes solid particles and clarifies the oil. The extracted CSO was stored in dark, airtight containers to protect it from light and oxygen, which can cause the oil to go rancid or deteriorate. Chia oil was stored in a cool place until it was used in biochemical analyses.

2.3. Polyphenolic composition using LC-HRMS analysis

LC-HRMS studies were performed with a Thermo Orbitrap Q-Exactive mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), equipped with a Troyasil C18 column (150 × 3 mm i.d., 3 µm particle size) (Istanbul, Turkey). The mobile phase A was composed of 1% formic acid–water and the mobile phase B was 1% formic acid–methanol. The gradient programs were 0–1.00 min, 50% A and 50% B; 1.01–6.00 min, 100% B; and finally, 6.01–10 min, 50% A and 50% B [71]. The flow rate was 0.35 mL/min and the column temperature was 22°C for the mobile phase. According to the literature, one of the most suitable solvent systems for HPLC was determined to be acidified methanol-water gradient and was applied. Because the electrospray ionization (ESI) source provides one of the best ionizations for relatively polar and small compounds, we used the ESI source. The ion scanning range of the device was set to *m*/*z* 85 and 1500 in high-resolution mode and scanning was performed [72,73].

2.4. Determination of total phenolics

Total phenolic content of CSO was determined according to the Folin-Ciocalteu method [26]. First, CSO was prepared stock solution at a concentration (1 mg/mL). Then, 1 mL of this stock solution was transferred to a test tube and the volume was adjusted to 25 mL with distilled water. Then, 0.5 mL Folin-Ciocalteu reagent and 1.5 mL sodium carbonate (2%) were added. Samples were vortexed and incubated at room temperature for 30 min, then absorbance was measured at 760 nm. Distilled water was used as blank and control. A calibration curve of gallic acid was prepared, and the total phenolic content of CSO was determined from the linear regression equation of this curve. Results are given as gallic acid equivalents per mL of oil [27].

2.5. Determination of total flavonoids

Flavonoids, which originate from plants, are the group of polyphenolic compounds that are most abundant in the human diet and have antioxidant activity. Total flavonoid content in CSO was determined according to the procedure of Chang and coworkers [74] as previously reported [72]. To determine the total flavonoids in CSO, the mixture containing 1 mL of CSO, 0.1 mL of aluminum nitrate (10%), and 0.1 mL of aqueous potassium acetate (1.0 M) was diluted with 4.3 mL of ethanol. After the solution was incubated at room temperature for 30 min, its absorbance was measured spectrophotometrically at 415 nm. Distilled water was used as blank and control. A calibration curve of Quercetin was prepared for the standard chart, and the flavonoid contents were determined from the linear regression equation of the calibration curve. Results are given as quercetin equivalent per mL of oil [35].

2.6. Fe^{3+} reducing ability assays

A series of test tubes were prepared, each containing different volumes of CSO to achieve varying concentrations. Briefly, 0.1 mL of $FeCl_3$ solution was added to 0.1 mL of $K_3[Fe(CN)_6]$ solution, and 0.1 mL of the CSO solution was transferred to each tube. The tubes at room temperature were incubated for 20 min. After incubation, 0.1 mL of TCA solution was added to each tube to stop the reaction. The contents were thoroughly mixed. A portion (0.1 mL) of the mix was transferred to a new set of tubes. A total of 0.2 mL of sodium acetate buffer was added to each tube, followed by 0.2 mL of Perl's reagent. The contents thoroughly were mixed and allowed the tubes to stand for 10 min to develop the Prussian blue color. The absorbance of each solution was measured at 700 nm using a spectrophotometer [75].

2.7. Cu^{2+} reducing ability assays

As another reduction method, the CUPRAC method using copper ions and a organic compound neocuproine (2,9-dimethyl-1,10-phenanthroline) was applied [76]. The stock solution of CSO was mixed with the working reagent in a test tube. The reaction mixture was incubated in the dark for 30 min to allow the antioxidants in the sample to react with the Cu^{2+} ions and reduce them to Cu^{+}. The absorbance of the reaction mixture was measured at a specific 450 nm wavelength [77]. The intensity of the color formed is proportional to the reduced capacity of the antioxidants present in the sample.

2.8. FRAP reducing ability assays

The ferric reducing antioxidant power (FRAP) method, which also uses iron ions as the final reduction method, was performed. FRAP assay is a common method used to measure the antioxidant activity of various compounds, including plant extracts and oils like CSO. This assay measures the ability of an antioxidant to reduce a ferric ion (Fe^{3+}) to a ferrous ion (Fe^{2+}) in the presence of a reducing agent. The reduction of the ferric ion is accompanied by the formation of a colored Fe^{2+}-tripyridyltriazine (TPTZ) complex, which can be measured spectrophotometrically [78]. The FRAP reagent was prepared by mixing TPTZ (10 mM in 40 mM HCl) solution with $FeCl_3$ (20 mM) solution and acetate buffer (0.3 M, pH 3.6). In a test tube, FRAP reagent was added, and an appropriate volume of CSO sample and mixed well. The mixture at 37°C was incubated for a fixed period. During this time, the antioxidants in CSO reduced the ferric ions in the reagent, forming the colored ferrous-tripyridyltriazine complex. After the incubation period, the absorbance of the colored complex was measured at a specific wavelength of 593 nm [79].

2.9. DPPH radical scavenging activity

Two different methods were used to determine the radical scavenging activity of CSO. The DPPH assay is a widely used method to evaluate the antioxidant capacity of various compounds, including plant extracts, essential oils, and oils like CSO [80]. It measures the ability of an antioxidant to donate an electron or hydrogen atom to the stable DPPH radical, thereby reducing it to a non-radical form. The DPPH radical scavenging activity of CSO was determined using a spectroscopic assay. Small amounts of CSO solutions were mixed with the DPPH solution. The mixture was allowed to incubate in

the dark at room temperature for a specified period of time. During this incubation period, the CSO react with the DPPH radical, which caused the purple color of the DPPH solution to fade. After the incubation period, the decrease in absorbance (color intensity) of the DPPH solution was measured using a spectrophotometer at 517 nm. A lower absorbance indicates a higher radical scavenging activity of the CSO [81].

2.10. ABTS radical scavenging activity

Gulcin's method was used to determine the ABTS radical scavenging ability of CSO [82]. Initially, an aqueous solution of ABTS (7.0 mM) was prepared and this solution was reacted with $K_2S_2O_8$ (2.5 mM) to produce radical cation ($ABTS^{\cdot+}$). This produced cation was diluted with phosphate buffer (0.1 M, pH 7.4), and the absorbance value of the control was adjusted to a constant value at 734 nm. Then, 1 mL $ABTS^{\cdot+}$ solution was added to 3 mL of CSO at different concentrations and waited for 30 min. The residual absorbance of $ABTS^{\cdot+}$ was measured at 734 nm [83]. The radical scavenging activity, expressed as the percentage inhibition of DPPH and ABTS radicals, was calculated using the following formula:

$$\text{Radical scavenging activity}\,(\%) = \left(1 - \frac{\text{Absorbance of CSO}}{\text{Absorbance of control}}\right) \times 100$$

The data obtained from the experiment were used to plot a graph of percentage radical scavenging activity against the concentration of CSO. The concentration at which the oil scavenges 50% of the DPPH and ABTS radicals is known as the IC_{50} value. A lower IC_{50} value indicates higher antioxidant activity. These assays provide valuable information about the antioxidant potential of CSO and its ability to neutralize free radicals, which are associated with oxidative stress and various health issues [84].

2.11. Acetylcholinesterase inhibition assay

Acetylthiocholine iodide (AChI) and 5,5′-dithio-bis-(2-nitroben zoic acid) (DTNB) were used as substrates of acetylcholinesterase enzyme (AChE) [85]. Firstly, 1 mL of Tris/HCl buffer (1.0 M, pH 8.0), 10 µL of different concentrations of CSO, and 50 µL AChE were mixed in a test tube. Then, the sample was incubated at 25°C for 15 min and 50 µL of DTNB solution (0.5 mM) was added. The reaction was started by transferring 50 µL of AChI solution (10 mM), and absorbance was measured at 412 nm.

2.12. α-Amylase inhibition assay

The Xiao procedure was used to determine the effect of CSO on inhibiting the α-amylase enzyme [86]. First, the substrate of the enzyme was prepared by mixing 1 g of starch with 50 mL of NaOH solution (0.4 M). The mixture was heated at 80°C for 20 min and allowed to cool. The pH of the solution was then adjusted to 6.9 and the volume to 100 mL using distilled water. Then, 35 µL starch solution, 35 µL phosphate buffer (pH 6.9) and 5 µL CSO solution were mixed. After 20 min of incubation at 37°C, 20 µL α-amylase solution was added and incubated again for 20 min. The reaction was completed by adding 50 µL HCl (0.1 M), and absorbance was measured at 580 nm.

2.13. Human carbonic anhydrase II isoenzyme (hCA II) inhibition assay

The human carbonic anhydrase II isoenzyme (hCA II) purified from human erythrocyte cells was used to determine the inhibitory effect of CSO on the hCA II [87]. For inhibition studies, esterase activity method was used. Esterase activity assays are commonly performed to determine the enzymatic activity of carbonic anhydrases, including hCA II, using various ester substrates. The inhibition effect was investigated by applying different concentrations of CSO. The ester substrate (p-nitrophenyl acetate) solution was prepared and disolved in an organic solvent (DMSO) to make a concentrated stock solution. The working solution of the ester substrate was prepared by diluting the stock solution with the buffer solution. Small amount of hCA II isoenzyme was added to the buffer solution, and absorbance was measured at 348 nm.

2.14. Statistical analysis

Statistical analyses were performed using Student's *t*-test (GraphPad Prism 6, GraphPad, La Jolla, CA, USA, Software 7.0). The data are presented as means ± standard deviations (SD). The minimum significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Total phenolic and flavonoid contents

Phenolic compounds are widely distributed in plants and had a vital role in human diet and health. They act as reducing agents in biological systems due to their antioxidant power [88]. Total phenolic content of CSO was determined according to the Folin-Ciocalteu method. Gallic acid equivalent from the linear regression equation was obtained from different gallic acid concentrations (Absorbance = 0.009 × [GAE], r^2: 0.9988). The total phenolic content in 1 mL of CSO was determined to be 784.44 µg GAE/mL. The obtained results emphasized that the CSO is a good source of phytochemicals content. The current study affirms the earlier studies about the high phenolic content of CSO as shown by Chen et al. [89] who revealed the phenolic content of chia seeds is 35.21–57.75 mg GAE/kg. The phenolic content of white chia seeds (3.52 ± 0.08 mg GAE) was significantly higher than those of black seeds (3.42 ± 0.06 mg GAE/g) [90].

Flavonoids are the most common group of polyphenolic compounds found in the human diet. They are abundant in plants and plant-derived products. They also have many biological effects such as antioxidant, antiviral and antimutagenic abilities. Quercetin, a flavonoid molecule that we used as a reference in our study, is a well-known polyphenol of plant origin with anti-inflammatory and antioxidant properties [47]. Flavonoids could chelate metals by forming complexes with metal ions [81]. Total flavonoids in CSO was calculated according to the aluminum nitrate method. For the determination of total flavonoids in CSO, a quercetin equivalent from the linear regression equation was obtained from different quercetin concentrations (Absorbance = 0.0033 − 0.012 × [QE], r^2: 0.9912). The total flavonoid content of CSO was determined to be 150.00 µg QE/mL.

3.2. Polyphenolic composition of CSO

Studying herbal extracts holds significant importance in both traditional and modern medicine due to their potential therapeutic benefits and their cultural and historical significance. Herbal medicine has been used for centuries in various cultures around the world. Traditional herbal knowledge has been passed down through generations and is often deeply rooted in cultural practices. Studying herbal extracts allows us to preserve and understand these valuable traditions, ensuring that ancient wisdom is not lost and can be integrated into modern healthcare systems. Many modern medicines are derived from natural sources, including plants [91]. Herbal extracts often contain a complex mixture of

Table 1
Chemical composition and validation parameters of chia (*Salvia hispanica*) seeds (CSO) (mg/L oil) obtained using LC-HRMS.

Compounds	RT	Molecular Formula	*m/z*	Ionization Mode	Linear Range	Linear Regression Equation	LOD/ LOQ	R^2	Recovery	U%	Phenolics
Ascorbic acid	1.99	$C_6H_8O_6$	175.0248	Negative	0.5–10	y = 0.00347x – 0.00137	0.39/ 1.29	0.9988	96.20	3.94	<LOD
Epigallocatechin	2.15	$C_{15}H_{14}O_7$	307.0812	Positive	0.3–5	y = 0.00317x + 0.000443	0.17/ 0.57	0.9947	102.22	3.09	3.59
Chlorogenic acid	2.43	$C_{16}H_{18}O_9$	353.0878	Negative	0.05–10	y = 0.00817x + 0.000163	0.02/ 0.06	0.9994	96.68	3.58	0.26
Fumaric acid	2.45	$C_4H_4O_4$	115.0037	Negative	0.1–10	y = 0.00061x – 0.0000329	0.05/ 0.17	0.9991	97.13	2.88	<LOD
Verbascoside	2.89	$C_{29}H_{36}O_{15}$	623.1981	Negative	0.1–10	y = 0.00758x + 0.000563	0.03/ 0.10	0.9995	96.19	2.93	0.02
Orientin	3.09	$C_{21}H_{20}O_{11}$	447.0933	Negative	0.1–10	y = 0.00757x + 0.000347	0.01/ 0.03	0.9993	96.22	3.67	0.12
Caffeic acid	3.17	$C_9H_8O_4$	179.0350	Negative	0.3–10	y = 0.0304x + 0.00366	0.08/ 0.27	0.9993	94.51	3.74	0.15
Luteolin-7-rutinoside	3.85	$C_{27}H_{30}O_{15}$	593.1512	Negative	0.1–10	y = 0.00879x + 0.000739	0.01/ 0.03	0.9988	93.05	3.06	0.07
Luteolin 7-glycoside	4.24	$C_{21}H_{20}O_{11}$	447.0933	Negative	0.1–7	y = 0.0162x + 0.00226	0.01/ 0.03	0.9961	96.31	4.14	0.12
Rutin	4.48	$C_{27}H_{30}O_{16}$	609.1461	Negative	0.05–10	y = 0.00329x – 0.00005576	0.01/ 0.03	0.999	96.97	3.07	<LOD
Rosmarinic acid	4.66	$C_{18}H_{16}O_8$	359.0772	Negative	0.05–10	y = 0.00717x – 0.0003067	0.01/ 0.03	0.9992	99.85	3.77	1.33
Hyperoside	4.58	$C_{21}H_{20}O_{12}$	463.0882	Negative	0.05–10	y = 0.0072x – 0.00003096	0.01/ 0.03	0.9995	96.62	3.46	0.07
Apigenin 7-glycoside	5.01	$C_{21}H_{20}O_{10}$	431.0984	Negative	0.3–7	y = 0.0246x + 0.00306	0.01/ 0.03	0.9962	96.07	2.86	<LOD
Ellagic acid	5.12	$C_{14}H_6O_8$	300.9990	Negative	0.05–10	y = 0.0085x – 0.000612	0.03/ 1.00	0.9994	101.49	3.59	0.12
Quercitrin	5.13	$C_{21}H_{20}O_{11}$	447.0933	Negative	0.05–10	y = 0.0179 + 0.0003331	0.01/ 0.03	0.999	97.00	4.20	0.89
Herniarin	5.64	$C_{10}H_8O_3$	177.0546	Positive	0.1–7	y = 0.309x + 0.0266	0.01/ 0.03	0.9983	92.92	2.95	0.05
Quercetin	5.68	$C_{15}H_{10}O_7$	301.0354	Negative	0.1–10	y = 0.0509x + 0.00467	0.01/ 0.03	0.9978	96.41	3.78	<LOD
Salicylic acid	5.72	$C_7H_6O_3$	137.0244	Negative	0.3–10	y = 0.0361x + 0.00245	0.01/ 0.03	0.9982	92.88	3.89	<LOD
Naringenin	5.74	$C_{15}H_{12}O_5$	271.0612	Negative	0.1–10	y = 0.0281x + 0.00182	0.01/ 0.03	0.9995	86.65	1.89	0.48
Luteolin	5.84	$C_{15}H_{10}O_6$	285.0405	Negative	0.1–10	y = 0.117x + 0.00848	0.01/ 0.03	0.9981	96.98	4.20	2.19
Apigenin	6.2	$C_{15}H_{10}O_5$	269.0456	Negative	0.3–10	y = 0.104x + 0.0199	0.01/ 0.03	0.9998	81.55	3.42	1.41
Hispidulin	6.24	$C_{16}H_{12}O_6$	301.0707	Positive	0.05–10	y = 0.02614x + 0.0003114	0.01/ 0.03	0.9993	98.36	2.87	2.25
Isosakuranetin	6.58	$C_{16}H_{14}O_5$	285.0769	Negative	0.05–10	y = 0.0235x + 0.000561	0.01/ 0.03	0.9992	96.56	3.41	29.07
Penduletin	6.64	$C_{18}H_{16}O_7$	343.0823	Negative	0.3–10	y = 0.0258x + 0.00253	0.01/ 0.03	0.9991	83.43	3.20	0.81
CAPE	6.66	$C_{17}H_{16}O_4$	283.0976	Negative	0.3–7	y = 0.255x + 0.0477	0.01/ 0.03	0.9964	94.42	3.13	0.03
Chrysin	6.99	$C_{15}H_{10}O_4$	253.0506	Negative	0.05–7	y = 0.0964x – 0.0002622	0.01/ 0.03	0.999	87.92	3.24	0.90
Quillaic acid	7.44	$C_{30}H_{46}O_5$	485.3273	Negative	0.05–10	y = 0.00781x – 0.0001318	0.01/ 0.03	0.9992	90.29	2.56	0.88
Caryophyllene oxide	8.82	$C_{15}H_{24}O$	221.1900	Positive	0.3–7	y = 0.00151x + 0.00692	0.10/ 0.50	0.9909	96.87	4.05	>LOQ

bioactive compounds such as alkaloids, flavonoids, terpenoids, and phenolic compounds. These compounds can have a wide range of pharmacological effects, including anti-inflammatory, antioxidant, antimicrobial, and anticancer properties. By studying herbal extracts, researchers can identify and isolate these bioactive compounds for further investigation and potential drug development [92].

In this study, which was carried out with the LC-HRMS method, 28 different phenolic structures that are likely to be found in vegetable oils were investigated. LC-HRMS results showed that the major phenolic compound in the structure of CSO is isosakuranetin (29.07 mg/L oil). It has been determined that there are approximately 10 times more isosakuranetin than other phenolic compounds in the structure of CSO. It was also found that CSO is rich in terms of epigallocatechin (3.59 mg/L oil), hispidulin (2.25 mg/L oil) and luteolin (2.19 mg/L oil) when compared to other compounds (Table 1). On the other hand, the amount of 7 (ascorbic acid, fumaric acid, rutin, apigenin 7-glycoside, quercetin, salicylic acid and caryophyllene oxide) of the studied phenolic compounds remained below the detectable amount. With these results, in our study, we observed that there is a positive correlation between phenolic content and antioxidant activity of CSO. Also, in our recent studies, the most abundant phenolic compounds in cinnamon (*Cinnamomum zeylanicum*) leaf oil were found as hispidulin (9.98 mg/L oil), herniarin (7.82 mg/L oil), and apigenin (6.61 mg/L oil) [72]. Also, the LC-HRMS findings revealed that apigenin

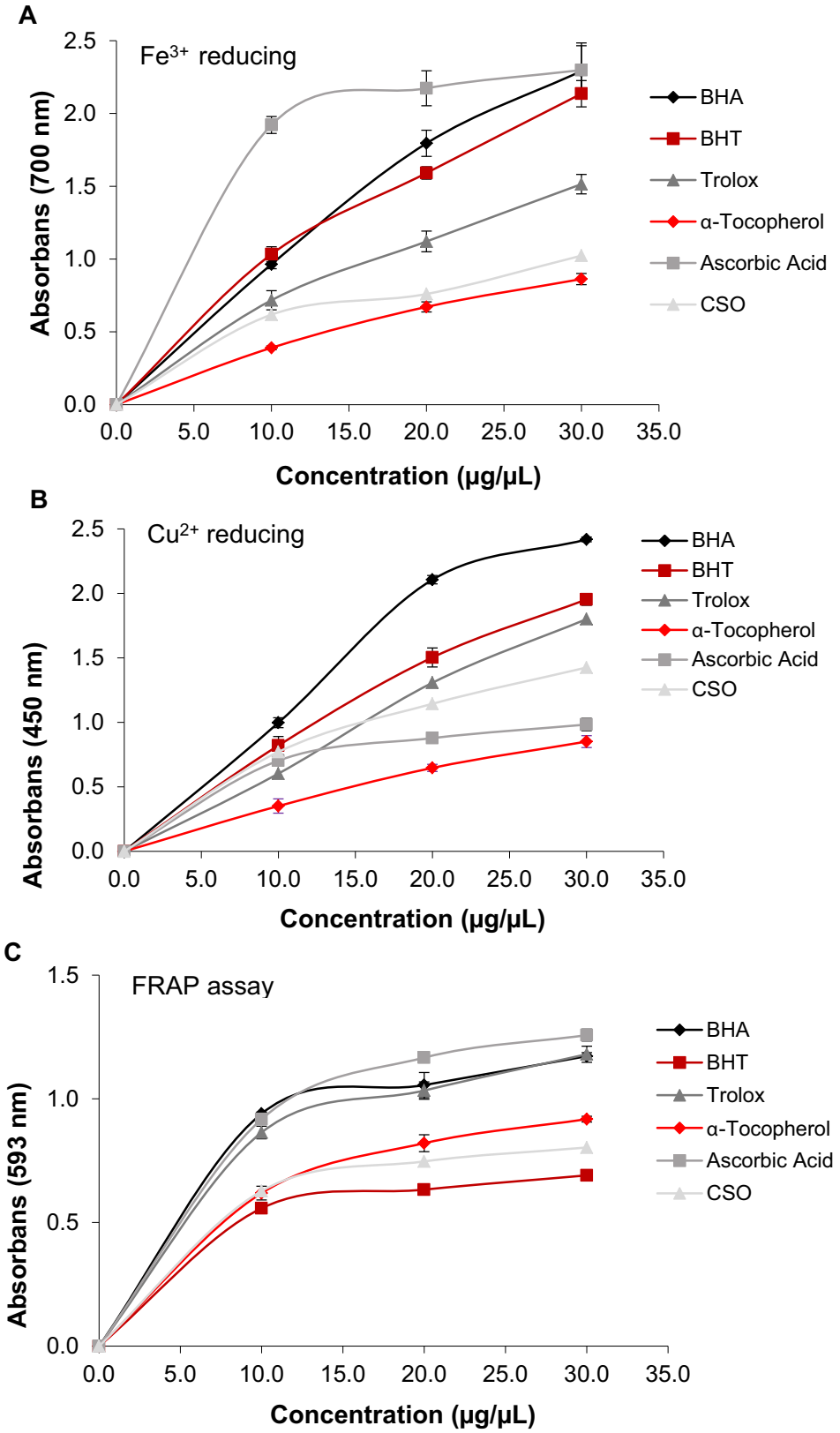

Fig. 1. (A) Ferric ions (Fe^{3+}) reducing ability of chia (*Salvia hispanica*) seeds (CSO) and standards; (B) Cupric ions (Cu^{2+}) reducing ability of chia (*Salvia hispanica*) seeds (CSO) and standards; (C) Ferric reducing power antioxidant activity of chia (*Salvia hispanica*) seeds (CSO) and standards.

Table 2
Reducing abilities of chia (*Salvia hispanica*) seeds (CSO) and standard antioxidants at 50 μg/mL concentration.

Antioxidants	Fe^{3+} reducing		Cu^{2+} reducing		Fe^{3+}-TPTZ reducing	
	λ_{700}	r^2	λ_{450}	r^2	λ_{593}	r^2
BHA	2.292 ± 0.012	0.9993	2.418 ± 0.018	0.9887	1.172 ± 0.014	0.9605
BHT	2.136 ± 0.090	0.9957	1.953 ± 0.045	0.9998	0.690 ± 0.008	0.9645
Trolox	1.514 ± 0.066	0.9963	1.800 ± 0.096	0.9974	1.180 ± 0.032	0.9732
α-Tocopherol	0.862 ± 0.038	0.9996	0.851 ± 0.046	0.9994	0.918 ± 0.011	0.9904
Ascorbic acid	2.298 ± 0.086	0.9659	0.983 ± 0.048	0.9822	1.257 ± 0.024	0.9869
CSO	1.023 ± 0.019	0.9684	1.425 ± 0.045	0.9759	0.803 ± 0.011	0.9760

(74.24 mg/L oil), epigallocatechin (12.89 mg/L oil), caryophyllene oxide (12.89 mg/L oil) are the most plentiful phenolics in kiwifruit (*Actinidia deliciosa*) oil kiwifruit oil [36]. In another study, apigenin (14.60 mg/L oil), quillaic acid (4.21 mg/L oil), and ascorbic acid (4.21 mg/L oil) were recorded as the most abundant phenolics in hemp (*Cannabis sativa*) seed oil [73].

LC-HRMS is a powerful analytical technique used in chemistry, biochemistry, and various scientific fields for the identification, quantification, and structural elucidation of compounds present in complex mixtures. It combines two main components: liquid chromatography (LC) and high-resolution mass spectrometry (HRMS). The determination of phenolic compounds in plant structures using the LC-HRMS method is an analytical approach widely used in phytochemical analysis. Phenolic compounds are a diverse group of secondary metabolites found in plants that play important roles in various biological activities, including antioxidant, anti-inflammatory, and antimicrobial properties. The LC-HRMS method offers a powerful and sensitive way to identify and quantify these compounds in plant samples. In recent years, LC-HRMS has become a cornerstone technique in the field of plant metabolomics and natural product research, enabling the discovery and quantification of a myriad of phenolic compounds in various plant species. This information is not only valuable for understanding the chemical composition of plants but also for exploring potential bioactive compounds with therapeutic applications. The findings in the current study are in line with the previous work of Chen et al. [89] who identified and quantified seventeen phenolic compounds in CSO using UPLC-QTOF-MS. Moreira et al. [93] revealed that chia seeds are a good source of phenolic compounds that can benefit the health of consumers as part of a healthy diet.

3.3. Antioxidant results

Three different reducing methods (iron reduction, copper reduction and FRAP) were used to understand the metal reduction potential of CSO. In the used methods, CSO was compared with five different antioxidant standard compounds (BHT, BHA, Trolox, α-Tocopherol and ascorbic acid). It can be said that CSO is a strong metal reducer and therefore has high antioxidant properties, since CSO exhibits more reduction potential than some standard antioxidants in all three methods.

If we consider the reduction method first, it is seen that CSO can convert more Fe^{3+} ions to Fe^{2+} ions than α-tocopherol. In this method, CSO and other antioxidant compounds at a concentration of 30 μg/mL (r^2 = 0.9719) exhibited reducing potential ($p < 0.01$) in the following order: Ascorbic acid > BHA > BHT > Trolox > CSO (1.023 ± 0.020) > α-Tocopherol (Fig. 1A and Table 2). As a second method, the results of CUPRAC show us that CSO has a higher reduction capacity of Cu^{2+} ions to Cu^{+} ions than both α-Tocopherol and ascorbic acid. In CUPRAC method, CSO and other standard compounds at a concentration of 30 μg/mL (r^2 = 0.9566) exhibited reducing potential ($p < 0.01$) in the following order: BHA > BHT > Trolox > CSO (1.425 ± 0.045) > Ascorbic acid > α-Tocopherol (Fig. 1B and Table 2). The fact that CSO has a higher antioxidant potential than both vitamins, which have important roles in living structures, can be considered as a valuable result. Finally, when looking at the FRAP method, it can be seen that CSO exhibits a higher antioxidant activity than BHT. In FRAP method, CSO and other standard compounds at 30 μg/mL (r^2 = 0.8199) exhibited effective reducing potential ($p < 0.01$) in the following order: Ascorbic acid > BHA > Trolox > α-Tocopherol > CSO (0.803 ± 0.012) > BHT (Fig. 1C and Table 2). BHT is an important artificial antioxidant and is widely used in many different fields.

The free radical scavenging ability of CSO was demonstrated by tests on two different radical structures (DPPH and ABTS). The same standard antioxidant compounds were used in the radical scavenging measurements as in the metal reduction methods. IC_{50} values were calculated by drawing graphs for both CSO and other antioxidant compounds. The smaller the IC_{50} value, the greater the radical scavenging efficiency [94].

All standard compounds showed higher activity than CSO (IC_{50}: 39.00 μg/mL, r^2: 0.9018) in ABTS scavenging tests (Fig. 2A). In DPPH radical scavenging tests, on the other hand, CSO showed higher radical scavenging activity than BHT (Fig. 2B) alone (IC_{50}: 30.30 μg/mL, r^2: 0.9036 for CSO, IC_{50}: 49.50 μg/mL, r^2: 0.9957 for BHT). Trolox (IC_{50}: 6.03 μg/mL, r^2: 0.9925) showed the highest activity in the DPPH scavenging method, while BHA (IC_{50}: 6.35 μg/mL, r^2: 0.9746) showed the highest activity in the ABTS scavenging method (Table 3).

Antioxidants are compounds that can prevent or slow down the damage caused by these harmful molecules, which are implicated in various diseases and the aging process. Reduction methods play a crucial role in determining antioxidant activity, as they provide insights into the ability of a substance to counteract oxidative stress caused by ROS and free radicals [95]. These methods are a subset of antioxidant assays designed to assess the ability of a substance to neutralize metal ions and inhibit oxidative reactions involving these metals. In this study, the reducing capacity of CSO was evaluated using three different methods (FRAP, CUPRAC, and Fe^{3+} reducing). In all three methods, CSO exhibited higher reducing power than some standard compounds (α-Tocopherol, ascorbic acid, BHT). This result showed that CSO has significant antioxidant potential.

Radical scavenging methods involve the use of antioxidants to neutralize free radicals and prevent oxidative damage. These methods are crucial for enhancing the antioxidant power of substances, as they help combat the harmful effects of excess free radicals [96]. In our study, two different radicals were tested. The most remarkable result was in the DPPH radical scavenging study. The DPPH radical scavenging power of CSO was measured to be greater than that of BHT, an important standard antioxidant compound. This result again revealed that CSO has high antioxidant activity.

3.4. Enzyme inhibition abilities of CSO

Studying the inhibitory effects of plant extracts on important metabolic enzymes is a significant area of research with broad

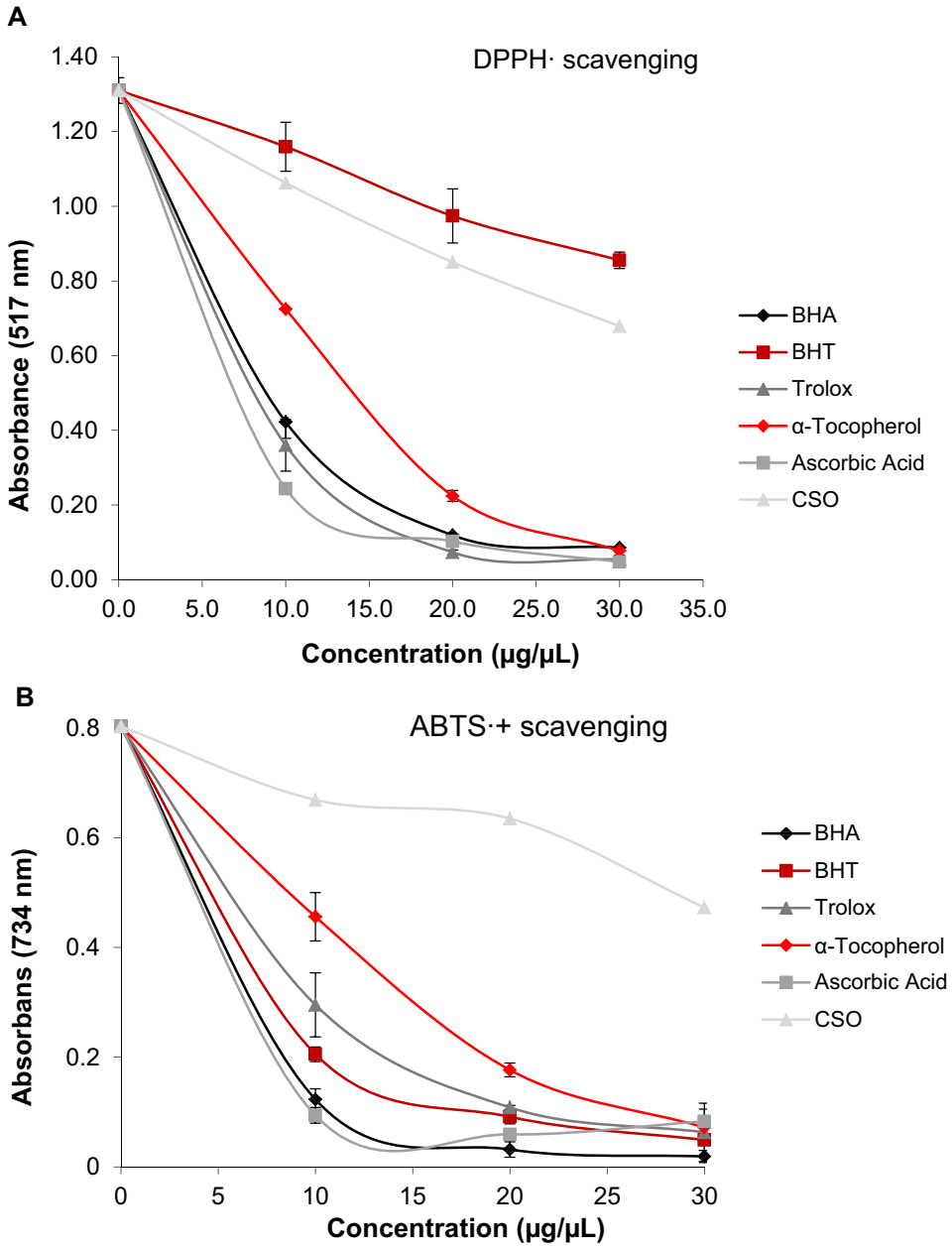

Fig. 2. (A) DPPH• radical scavenging activities of chia (*Salvia hispanica*) seeds (CSO) and standards; (B) ABTS•+ radical scavenging activities of chia (*Salvia hispanica*) seeds (CSO) and standards.

Table 3
DPPH• and ABTS•+ scavenging activities of chia (*Salvia hispanica*) seeds (CSO) and antioxidant standards.

Antioxidants	DPPH• scavenging		ABTS•+ scavenging	
	IC_{50} (µ g/mL)	r^2	IC_{50} (µ g/mL)	r^2
BHA	6.86	0.9949	6.35	0.9746
BHT	49.50	0.9957	12.60	0.9995
Trolox	6.03	0.9925	16.50	0.9775
α-Tocopherol	7.70	0.9961	18.72	0.9347
Ascorbic acid	5.82	0.9668	11.74	0.9983
CSO	30.30	0.9936	39.00	0.9018

implications for various fields including medicine, pharmacology, nutrition, and agriculture [97]. This line of research contributes valuable insights into the potential therapeutic benefits, drug development, and understanding of natural compounds. Enzyme inhibition properties of CSO were studied towards three important metabolic enzymes (α-amylase, acetylcholinesterase and carbonic anhydrase II).

The enzyme inhibition properties of CSO were studied for three important metabolic enzymes (α-amylase, AChE and CA II). Acarbose for α-amylase enzyme, Tacrine for AChE enzyme and acetazo-

Table 4
IC_{50} values of CSO against acetylcholinesterase, α-amylase, and carbonic anhydrase II enzymes.

Enzymes	CSO		Standard inhibitors	
	IC_{50} (μ g/mL)	r^2	IC_{50} (μ g/mL)	r^2
α-Amylase[a]	553.07	0.9058	7.54	0.9074
Acetylcholinesterase[b]	17.60	0.9874	8.82	0.9836
Carbonic anhydrase II[c]	243.24	0.9092	9.96	0.9930

[a] Acarbose was a standard inhibitor for α-glycosidase and α-amylase enzymes.
[b] Tacrine was used as a standard inhibitor for acetylcholinesterase.
[c] Acetazolamide was used as a standard inhibitor for carbonic anhydrase II.

lamide (AZA) for CA II enzyme were used as standard inhibitors [98]. IC_{50} values were calculated by means of the graphs obtained by making activity measurements at different concentrations for both CSO and standard inhibitors. Although CSO had an inhibitory effect on all three enzymes, a remarkable or in other words close to standard inhibitors, an IC_{50} value was found only on AChE enzyme. High IC_{50} values and therefore low inhibition effect of CSO were observed on α-amylase and CA II enzymes compared to standard inhibitors (Table 4).

Acetylcholine is a neurotransmitter that is involved in various functions in the central and peripheral nervous systems, including muscle contraction, cognition, memory, and autonomic functions. Proper regulation of its levels is essential for maintaining normal neural function [99]. Inhibition studies help researchers understand how AChE activity is controlled and how disruptions can lead to neurological disorders. Inhibition studies provide insights into the mechanisms of AChE inhibition, aiding in the development of new therapeutic agents [100].

The present study investigated the inhibitory effect of CSO on the AChE enzyme associated with AD. Different concentrations of CSO were administered and IC_{50} values were determined, and the results summarized in Table 4. It was demonstrated that CSO exhibited an excellent inhibition effect against AChE with an IC_{50} value of 17.60 μg/mL (r^2: 0.9874), while tacrine, as a standard cholinesterase inhibitor, showed the IC_{50} value of 9.96 μg/mL (r^2: 0.9930). The potential inhibition of AChE by natural bioactive compounds in plant-based extracts might open a promising perspective for the development of new drugs capable of delaying the progress of mental deterioration and therefore represents a rational therapeutic approach to the treatment of AD. The high AChE inhibitory effect of CSO might be due to it is high antioxidant activity or the ability of bioactive compounds to interact with the active site of the enzyme preventing the substrate to bind with.

Researchers can design compounds that selectively target AChE, potentially leading to more effective treatments for conditions like AD. In this study we conducted, it was observed that CSO significantly inhibited the AChE enzyme. This result is encouraging for drug development studies on AD. Because the inhibition value shown by CSO is lower than that of tacrine, which is the standard AChE inhibitor, but it is close to it. In another study conducted by our group, the inhibition IC_{50} value of cinnamon leaf oil on AChE was determined as 16.03 μg/mL. In our research, we also studied two other metabolic enzymes (α-amylase and CA II), but it was seen that CSO did not have a significant inhibitory effect on these enzymes. Because the standard inhibitors of both enzymes exhibited very advanced activity compared to CSO.

The α-amylase mainly occurrs in the saliva and pancreas helping carbohydrate hydrolysis. Many synthetic drugs acting as antidiabetics were designed to interact with the active site of α-amylase to inhibit the enzyme hydrolysis ability. On the other hand, these drugs may cause some negative implications for patients [101]. This issue is stimulating scientists to develop such natural α-amylase inhibitor with minimum or no side effects. CSO exhibited a moderate inhibitory ability against α-amylase with IC_{50} values of 533.07 μg/mL (r^2: 0.9058) compared to the standard inhibitory tacrine (IC_{50}: 7.54 μg/mL r^2: 0.9074). IC_{50} of CSO needed for inhibition of α-amylase is greater than that for aAChE (16.03 μg/mL) and CA II (IC_{50}: 243.24 μg/mL) (Table 4). It suggests the low ability of CSO to inhibit α-amylase activity. The previous study of Tavera-Hernández et al. reported the high ability of chia peptides for inhibition of the α-amylase enzyme (79.19–85.61%) [102]. Similarly, several bioactive compounds based-plant extracts show a high inhibitory power against α-amylase [103].

4. Conclusions

The antioxidant activity of CSO prepared by the cold press method was evaluated using different methods. CSO was found to have significant antioxidant potential. The current study showed the high phenolic and flavonoid content of chia seed oil. The antioxidant activity of CSO was also evaluated. The results showed that CSO had a high level of antioxidant activity. Therefore, CSO could be a promising natural source of antioxidants and polyphenols. In addition, the inhibition effect of CSO on three different metabolic enzymes was studied. It was found that CSO had a high inhibitory effect on the AChE enzyme, but a low inhibitory effect on α-glycosidase and hCA II enzymes. The results show that CSO can be a rich and useful source of biologically important biomolecules. In the content screening made by the LC-HRMS method, it was determined that isosakuranetin was the major compound in the structure of the plant. Isosakuranetin has shown potential medicinal properties, such as antioxidant and anti-inflammatory effects. It may also have neuroprotective properties and could be explored for its therapeutic potential in the treatment of neurodegenerative diseases. Further research is needed to fully understand the extent of its medicinal benefits and its possible applications in healthcare. The high antioxidant properties of CSO make it a promising candidate for various applications in the food and pharmaceutical industries. CSO could be used as a natural preservative to extend the shelf life of food products, or as an ingredient in dietary supplements to promote overall health and well-being. Additionally, the strong reduction exhibited by CSO suggests its potential in mitigating oxidative stress-related diseases and disorders. CSO had a high inhibitory effect on AChE, but a low inhibitory effect on α-glycosidase and hCA II. The high inhibitory effect of CSO on AChE may have significant implications in the field of medicine. AChE is an enzyme that plays a crucial role in the breakdown of ACh, a neurotransmitter involved in various physiological processes. By inhibiting this enzyme, CSO could potentially be used to modulate acetylcholine levels and treat conditions such as AD, where ACh deficiency is a characteristic feature.

CRediT authorship contribution statement

Muzaffer Mutlu: Methodology, Investigation. **Zeynebe Bingol:** Methodology, Investigation. **Eda Mehtap Ozden:** Methodology, Investigation. **Ekrem Köksal:** Methodology, Investigation. **Adem Erturk:** Methodology, Investigation. **Ahmet C. Goren:** Methodol-

ogy, Investigation. **Saleh Alwasel:** Writing – review & editing, Supervision. **İlhami Gulcin:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare no conflict of interest.

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Supplementary material

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Data availability

The data that have been used are confidential.

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