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Evaluation of the Antioxidant, Antidiabetic and Anti-Alzheimer Effects of *Capsella bursa-pastoris*-Polyphenolic Profiling by LC-MS/MS

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Abstract: *Capsella bursa-pastoris* species of the *Capsella* herb were examined in this study for sreening of antioxidant, antidiabetic and anti-Alzheimer effects. Traditionally, people consumed *C. bursa-pastoris* against famine and satisfy their hunger. *C. bursa-pastoris* species have been shown to have utility as food, medicine, and industrial materials after extensive investigation. The antioxidant properties of methanol and water extracts of *C. bursa-pastoris* species were assessed using; Fe^{3+}-2,4,6-tris(2-pyridyl)-*S*-triazine (TPTZ), ferric (Fe^{3+}), and cupric (Cu^{2+}) iobs reducing assays, as well as 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid radical ($ABTS^{\bullet+}$) and N,N-dimethyl-p-phenylenediamine (DMPD) radical scavenging activities. The antioxidant and reducing capacity of *C. bursa-pastoris* species aerial parts water and methanol extracts were compared with standard antioxidants. The phenolic and flavonoid contents in methanol and water extracts of *C. bursa-pastoris* species were measured 6.86 to 12.00 mg GAE/g and 61.67 to 145.0 mg QE/g, respectively. The inhibitions of effects of water and methanol extract of *C. bursa-pastoris* species against α-amylase, and acetylcholinesterase (AChE) enzymes were investigated. The IC_{50} values were found as 168.6 to 238.6 µg/mL against α-amylase and 19.0 to 20.9 µg /mL against AChE. The number of fenolik compounds in both extracts of *C. bursa-pastoris* species were recorded using LC-MS/MS. The major phenolic and flavonoid components detected in methanol extract of *C. bursa-pastoris* were quinic acid (22.629 mg/g), chlorogenic acid (3.211 mg/g), rutin (1.930 mg/g), hesperidin (0.893 mg/g), and isoquercitrin (0.783 mg/g). In a similar order, quinic acid (11.356 mg/g), cyranoside (9.463 mg/g), chlorogenic acid (6.072 mg/g), hesperidin (5.912 mg/g), and isoquercitrin (5.364 mg/g) were found as plentyfull pehenolic antioxidants in methanolic extract. The results clearly demonstrated that polyphenolic antioxidants-rich ingredients of the aerial parts of *C. bursa-pastoris* species are biological phenolic comounds have persuasive usage in the medication of diabetes and Alzheimer's diseases.

Keywords: *Capsella bursa-pastoris*; enzyme inhibition; antioxidant activity; α-amylase; acetylcholinesterase; phenolic compounds.

1. Introduction

Capsella bursa-pastoris L. (Brassicaceae) is a biennial or annual plant referred to as Lady's purse, Shepherd's purse or Shepherd's bag. In Türkiye, Central America, Eastern of Europe, North Africa and

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other Asian nations, it has been discovered and they are home to *C. bursa-pastoris* [1]. Some countries have employed the newly developed leaves and roots of the native species *C. bursa-pastoris*. Medic is an edible vegetable that may be consumed raw or cooked [2]. *C. bursa-pastoris* has a traditional usage history worldwide. It is commonly referred to as the shepherd's purse. Acetylcholine, tyramine, histamine, and choline are of great importance in the apical portions. The herb's phytochemical makeup has several health advantages, including hepatoprotective, antioxidant, cardiovascular, tranquilizer, anti-inflammatory, and anticancer actions [3]. Since ancient times, the herbaceous plant species *C. bursa-pastoris* has been employed as an aphrodisiac and laxative, demonstrating its therapeutic properties. It has healing, hemostatic, and uterine tonic properties in traditional medicine [4]. The compounds found in *C. bursa-pastoris* include sterols, fatty acids, amino acids, alkaloids, and flavonoids. Anti-inflammatory, anti-cancer, antioxidant, and effective versus metabolic illnesses are only a few of the positive health effects that are thought to be provided by the variation in its constitution [5]. The flavonoid components including quercetin, chrysoeriol, kaempferol, and isorhamnetin are responsible for the strong antioxidant activity of *C. bursa-pastoris*. According to in vitro research, its extracts contain antioxidant properties that stop the production of many types of free radicals, including hydrogen peroxide, peroxyl radicals, and hydroxyl radicals [6]. Plants have many different secondary metabolites with different structural variants, which leads to a variety of biological capabilities. The ability to exhibit antioxidant effects is especially present in phenolic ingredients with a series of hydroxyl groups [7].

Reactive oxygen species (ROS) are an inevitable consequence of the metabolism of oxygen. When oxidative stress conditions are present, ROS levels can rise sharply, damaging proteins, lipids, and DNA and causing deadly lesions like cancer, cardiovascular disease, and aging [8]. It was previously mentioned that prolonged exposure to several prooxidant factors, either directly or indirectly, has detrimental consequences on human health. The occurrence of chronic illnesses brought on by oxidative stress is significantly influenced by the modern lifestyle, which comprises eating refined nutriments, not exercising, and be liable to various types of chemicals and xenobiotics. Antioxidants are required to limit this impairment, and they should be included in everyday life either directly with supplements or through food [9]. The inactivation of ROS generation is balanced by the living antioxidant systems. When ROS is produced in excess under pathogenic conditions, oxidative stress results. ROS is generated when the body's natural antioxidant defenses aren't enough. The imbalance of ROS and antioxidant defense systems imbalance be concluded oxidative alteration of intracellular molecules or cellular membranes [10]. The defensive mechanism of the immune system includes antioxidant compounds and antioxidant enzymes. Both antioxidant systems reduce, halt, or stop the oxidation of certain biomolecules. They involve polyphenols and are strong ROS inhibitors that can successfully counteract their negative and unwanted effects [11]. However, antioxidant foods or supplements can minimize oxidative damage caused by ROS and free radicals in the body [12]. Numerous antioxidants present in plants have been discovered so far, including free radicals and ROS scavengers. Since synthetic antioxidants have grown less popular because of unfavorable side effects of them, there has been a sharp increase in the search for naturally occurring antioxidants in plants to utilize in meals and treatments [13]. To enhance the quality, natural flavonoids can be collected and utilized in the food business in place of synthetic ones. Because of their ability to inhibit lipids peroxidation, enhance the nutritive content, and lessen toxicity, flavonoids as natural antioxidant has gained popularity in recent years because of restrictions placed on the use of synthetic antioxidants [14]. Fruits, vegetables, and other natural foods contain a large spectrum of antioxidant molecules. Antioxidants, which derived from these sources can readily eliminate ROS in this situation [15]. Up till now, a lot of research has been conducted on medicinal herbs. The therapeutic herbs include fruits and plants that have a high phenolic content [16]. The various ways of action of phenolic compounds consist of the binding of electrophiles, the inactivation of ROS, the angiogenesis inhibition, the H-donation, and the inhibition of DNA oxidation [17]. As secondary metabolites, phenolic compounds are present in plants and protect methabolism against oxidative stress and progressive illnesses such as cancer, heart disease, cataracts, diabetes, and hypercholesterolemia [18].

Alzheimer’s disease (AD) is a significant health concern worldwide. There are several different treatment methods for AD. Among them, the inhibition of acetylcholinesterase (AChE) and butrylcholinesterase (BChE)are the most useful techniques [19]. Lipid peroxidative damage is associated with diverse ailments, including AD, coronary atherosclerosis, carcinogenesis, and other age-related ailments. Physiologically active compounds with antioxidant properties can help prevent this oxidative

damage. The prophylactic treatment of AD neurological problems still includes AChE and BChE activity measures [20]. Several different AChE inhibitors are utilized to treat AD signs. Some AChE inhibitors utilized in AD include galantamine, rivastigmine, donepezil, and tacrine. Additionally, their side effects include hepatotoxicity and gastrointestinal disturbance curtail the use of these drugs [21]. AD begins with memory loss and other progressive cognitive problems. Acetylcholine (ACh) insufficiency, inflammation, and oxidative stress are thought to be connected. Consumption of antioxidant-rich vegetables can thereby slow the course of AD and neurodegenerative disorders [22]. One effective therapy for these conditions is believed to be the inhibition of the vital enzymes connected to them. However, synthetic medications have a few unfavorable adverse effects. This problem was addressed by the investigators by establishing motivations to find alternative natural products [23]. In AD, bioactive compounds are used as AChE inhibitors (AChEIs) in clinical studies. AChEIs were also discovered to be phenolic compounds and were the first medications to treat AD [18].

Type-2 diabetes mellitus (T2DM) is a prevalent endocrine condition worldwide. The pathophysiology and categorization of T2DM include insulin activity resistance, lipid metabolism, aberrant glucose levels, and inadequate insulin production from pancreatic tissue β-cells [24]. The body struggles to get blood glucose to the cells, so the blood glucose level in diabetes individuals remains elevated as the cells begin to starve. Glucose levels after meals are also influenced by the breakdown and release of glucose from carbohydrates digestion [25]. The inhibition of the digestion enzymes including α-glycosidase and α-amylase represents one of the most prevalent remedies for T2DM. By delaying the absorption of glucose units, postprandial hyperglycemia, and postprandial plasma glucose concentration may be reduced [26].

The AChE enzyme will be inhibited to ascertain if *C. bursa-pastoris* methanol and water extracts have anti-AD properties. Similar experiments are being conducted to determine the IC_{50} rates of methanol and water extracts and examine the antidiabetic potential of plant extracts on α-amylase. To further understand the antioxidant activity of *C. bursa-pastoris*, tests such as metal ions reductions, DMPD and ABTS scavenging are performed, and the levels of phenolic and flavonoid compounds for methanol and water extracts were calculated. Phenolic ingredients determination utilizing the LC-MS/MS chromatography is used to determine the phenolic components that underpin the *C. bursa-pastoris*. AChE and α-amylase are linked to frequent and widespread health issues, and this study aimed to demonstrate how the *C. bursa-pastoris* extracts block these enzymes. According to the proposal, the data gathered from this study help to justify the necessity for more research to develop innovative dietary supplements and diets.

The purpose of this investigation was to determine the antioxidant potency of *C. bursa-pastoris*, which is used in remedy of prevalent ailments. Another goal was examination of inhibitory ability of *C. bursa-pastoris* extracts toward some metabolic enzymes associated with some global and common disseases.

2. Materials and Methods

2.1. Chemicals

Acetylcholinesterase, α-glycosidase enzymes and acetylcholine iodide, p-nitrophenyl-D-glucopyranoside, and phenolics of LC-MS/MS and other chemicals were taken from Merck or Sigma.

2.2. Plant Materials

The methanol and water extract of *C. bursa-pastoris* was dissolved in ethanol for the antioxidant activities. The plant taxa were taken during the flowering time from various phytogeographical regions of Turkey and recognized by Dr. Mehmet Cengiz Karaismailoğlu. The voucher samples are stored in the Siirt University Flora and Fauna Center (SUFAF) and Karaismailoğlu, M. C. collection. The examined *Capsella* taxa and their locations were given; *C. bursa-pastoris* Artvin, Koyuncular, roadsides, sloping hills, 900 m, May 22, 2017, and stored as SUFAF 5002.

2.3. Preparation Plant Extracts

Process of the extraction was followed, as stated before [27]. The samples water extracts were assembled applying 100 mL of distilled water and 25 g of dried *C. bursa-pastoris* aerial parts that had been ground in a mill. Boiled sample on a magnetic stirrer shaking for 20 minutes. In a lyophilizator (Labconco, Freezone), the filtrates lyophilized at -50 °C and 5 mmHg of pressure. For the methanol extracts of the samples, 25 g of dried *C. bursa-pastoris* plant aeral parts were ground, mixed with 100 milliliters of methanol, and agitated for 60 minutes with utilize a magnetic stirrer. Following the filtering of the extracts, filtrates were gathered. A rotary evaporator was used to extract the methanol set to 50 °C. The prepared samples extracts were kept at 20 °C in a dark plastic bottle before to use in experimental investigations [28]. The methanol and water extract of *C. bursa-pastoris* was dissolved in ethanol for its antioxidant activity.

2.4. Contents of Total Phenolics

Analysis of the phenolic composition of *C. bursa-pastoris* water and methanol extracts were performed [29]. with the inclusion of slight methodological changes [27]. To the 1.0 mL of Folin-Ciocalteu reagent, add the necessary 0.5 mL of every extract. After that, 0.5 mL of 1% carbonate is thoroughly added to the solution to neutralize it. Following a two-hour dark incubation period, the absorbance at 760 nm is measured against a blank sample composed of distilled water. The phenolics contents were recorded by utilizing the equation of the curve for gallic acid. The milligrams of gallic acid equivalents (GAE) per gram of *C. bursa-pastoris extract* are the findings of the phenolic ingredient analysis.

2.5. Determination of Total Flavonoid Contents

Flavonoids are a pervasive class of polyphenolic chemicals found in a standard human diet and in a wide variety of plants. A described process [30] was applied to calculate the total flavonoid ingredients in *C. bursa-pastoris* specimens. 1.5 mL of 95% methanol and 0.5 mL of the methanol or water extract of *C. bursa-pastoris* specimen was blended. After adding 1.5 mL of 10% Al $(NO_3)_3$, 0.5 mL of CH_3COOK (1.0 M), and 2.3 mL of distilled water, the samples were vortexed. After that, the vortexed specimens were stored in the dark for forty minutes (at room temperature). The absorbances were taken at 415 nm. Blank were measured with using distilled water. Flavonoid content was estimated using the curve's equation. The results, known as quercetin equivalents (QE), are displayed as milligrams per gram of extracts from *C. bursa-pastoris*.

2.6. Sample Preperation for LC-MS/MS Method

A volumetric flask containing 5 mL of ethanol-water (50:50 v/v) is used to dissolve each 100 mg of *C. bursa-pastoris* water and methanol extracts. One milliliter of this mixture is subsequently put into another volumetric flask that has a 5 mL capacity. Next, methanol extract and 100 µL of *C. bursa-pastoris* water were mixed and the volume was diluted with a 50:50 v/v ethanol-water ratio. 10 µL of the sample is introduced into the LC-MS/MS after a 1.5 mL aliquot from the finished mixture is moved into a vial with a cap. The autosampler's specimens are maintained at 15 °C for the duration of the experiment [31].

2.7. Preparation of Test Solution and Chromatograph Conditions

For *C. bursa-pastoris* extracts, the LC-MS/MS analytical methodology used in the current investigation was created and refined [32]. As a reference, 53 phenolic standards were used.

2.8. Fe^{3+} Reducing Ability Assays

Methanol extract and water extract's of *C. bursa-pastoris* ability to reduce Fe^{3+} is calculated [33], as mentioned [34]. The absorbances of both extracts were recorded at 700 nm. As a blank sample, phosphate buffer solution is used.

2.9. Cu^{2+} Reducing Ability Assays

The Cu^{2+} reducing of *C. bursa-pastoris* extracts were measured based on the of method of Apak et al. [35] with detailed less modification [36]. Their absorbances at 450 nm were finally measured using

spectrophotometry. The blank sample was an acetate buffer solution [37].

2.10. Fe^{3+}-TPTZ Reducing Assays

The Fe^{3+}-TPTZ reduction in acidic solution is the basis for the FRAP reducing power. The absorbance of the *C. bursa-pastoris* extracts were recorded at 593 nm. A blank specimen was produced by employing phosphate buffer mixture [38].

2.11. $ABTS^{\bullet+}$ Scavenging Activity

ABTS is a rather stable free radical. $ABTS^{\bullet+}$ scavenging activity measurement is recorded using the Re et al method [39]. The quantity of $ABTS^{\bullet+}$ that remains at 734 nm was quantified spectrophotometrically after a predetermined period [40]. The $ABTS^{\bullet+}$ reagent was generated, stabilized and equilibrated at 30 °C, the experiments conducted temperature. Then, 3 mL of methanol and water extract of *C. bursa-pastoris* solutions in ethanol at various concentrations (10–30 g/mL) are blended with 1 mL of the $ABTS^{\bullet+}$ solution. The radical scavenging rate was calculated for each concentration in relation to a blank including zero scavengers. Its absorbance was measured after 30 minutes of mixing. To ascertain the degree of decolorization, the absorbance decrease as a proportion was utilized.

2.12. $DMPD^{\bullet+}$ Scavenging Activity

The radical removal capability of *C. bursa-pastoris* extracts against DMPD was measured [41] with slight changes [42]. $DMPD^{\bullet+}$ was formed according to method procedure. The reaction volume was adjusted after standard antioxidants or plant sample extracts were added at various amounts (10–30 µg/mL). The reaction mixture was promptly supplemented with a milliliter of $DMPD^{\bullet+}$ solution. After carefully mixing the reaction mixtures, they were kept in dark and incubated for fifty minutes. The absorbance was observed at 505 nm. The half maximum scavenging concentrations (IC_{50}, µg/mL) were used to express the radical scavenging findings [43].

2.13. Metal Chelating Assay

Fe^{2+} chelating of *C. bursa-pastoris* extracts by 2,2´-bipyridine is typically carried out [39]. Shortly, various amounts of the *C. busa-pastoris* methanol or water sample extract and standards were relocated to a blend of 0.25 mL $FeSO_4$ (2 mM). As a result, the association of the sample and Fe^{2+} ions were settled. Thus, the sample chelates Fe^{2+} ions. Subsequently, 1.5 mL of 0.2% bipyridyl mixture diluted in 1 mL of Tris-HCl solution (pH 7.4) and 0.2 M HCl were added to the reaction solution progressively. Incubating the sample mixture for 30 minutes, then 2.5 mL of ethyl alcohol and 0.63 mL of distilled water were applied. Absorbances were measured at 522 nm contrary to a blank composed of buffer [44].

2.14. AChE Inhibition Assay

C. bursa-pastoris extracts cholinergic enzymes inhibitory activities are carried out [45] as described before [46]. AChE from electric eel serum was used to achieve this. In summary, the enzyme quantity is mixed with a particular quantity of *C. bursa-pastoris* water and methanol extract samples. For ten minutes, the mixes were maintained at 20 °C. After that, 50 µL of solutions containing acetylthiocholine iodide (AChI) and 5,5′-dithio-bis (2-nitro-benzoic acid (DTNB) (0.5 mM) were applied. After starting the reaction medium, the absorbances of the reactions were recorded using spectrophotometry at 412 nm [47].

2.15. α-Amylase Inhibition Assay

α-Amylase inhibition effects of extracts from *C. bursa-pastoris* were examined [48]. In short, 1 g of starch is solved in 40 mL of 0.4 M alkaline mixture and boiled at 80 °C for 30 minutes. Once the liquid has cooled, the pH is corrected to 6.9, and the reaction volume is brought to 100 mL utilizing deionized water. The same volume (35 µL) of starch and phosphate buffer (pH 6.9) is then combined with varying quantities of the methanol and water extract. After that, the finished mixture is combined with 20 µL of α-amylase mixture and incubation of mixture was done for one hour at 35 ºC. By adding 50 µL of HCl (0.1 M) to terminate the reaction, the samples' absorbance at 580 nm is measured toward a blank sample that includes buffer.

2.16. Statistical Analysis

Three times are repeated for each experiment. Results were given as mean ± SD. The one-way ANOVA and Tukey's post hoc analysis was applied; $P<0.05$ was taken to show a statistically significant difference.

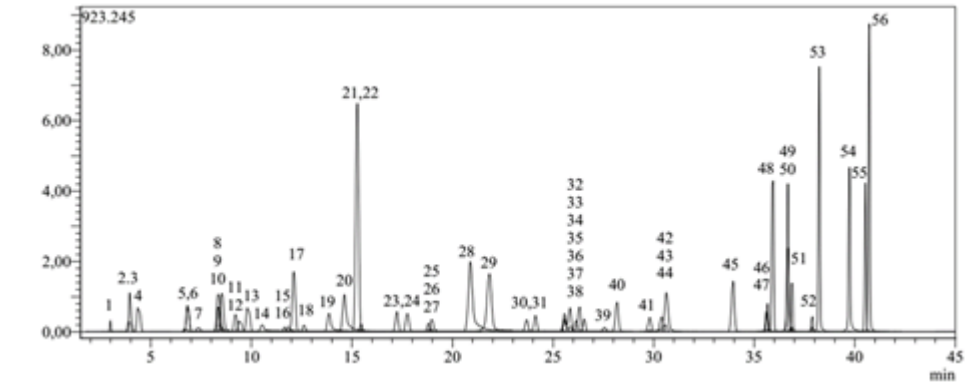
3. Results and Discussion

Phenolics are the common metabolites found in plants kingdom. These different classes of organic substances have garnered plenty of interest as possible herbal antioxidants because they are efficient metal chelators and radical scavengers. Surveys indicate that phenol's redox features, hydrogen givers, and singlet oxygen repellent are key contributors to the compound's antioxidative activity [49]. Phenolics in all plants are an essential and significant part of the diet of humans. Their biochemical effects, involve antioxidant features, has drawn a lot of interest to them [50]. Flavonoids are phenolic chemicals that have been extracted from a range of plants and have been shown to have antibacterial, antioxidant, and other positive effects in addition to their capacity to block light. Flavonoids' antioxidant activity has collected a lot of interest because of their capacity to either scavenge and inhibit the ROS creation [51]. The phenolics and flavonoids contents in water and methanol specimens of *C. bursa-pastoris* were determined as 6.86 to 12.00 mg GAE/g and 61.66 to 145.00 mg QE/g, respectively in this study (Table 1).The flavonoid contents of *C. bursa-pastoris* L. was found 13.76±0.29 mg/100 g DW in a regional study [52]. The plant's methanol extract revealed that *C. bursa-pastoris*, a wild vegetable from Pakistan's Potohar region, had a phenolic concentration of 1.56×10^{-4} mg GAE g^{-1} [53]. In this study, the extraction conditions and total phenolic and flavonoid contents of *C. bursa-pastoris* specimens were given at Table 1.

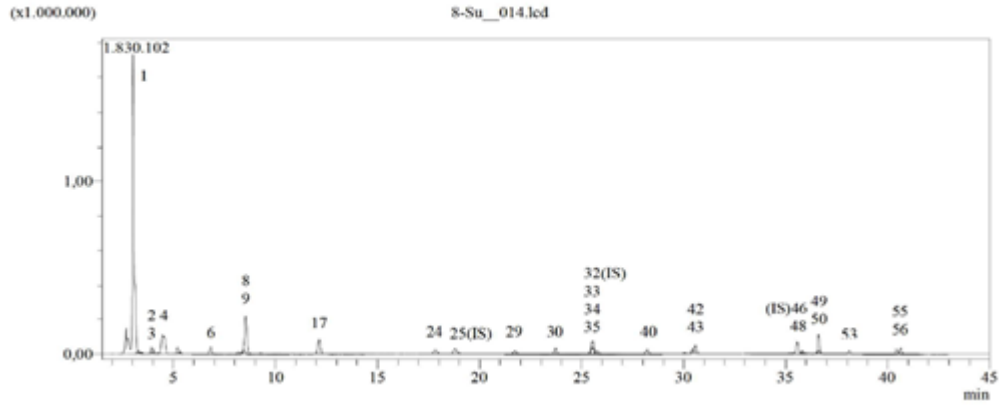
Table 1. The extraction properties and total phenolic and flavonoid ingredients in water and methanol extracts of *C. bursa-pastoris*.

Extraction properties	***C. bursa-pastoris***	
	Water extract	**Ethanol extract**
Dry aeral parts (g)	25	25
Solvent (mL)	Boiled water	Ethanol
Time (min)	30	60
Amount (g)	1.55	0.40
Yield (%)	6.2	1.6
Phenolic content (mg GAE)	12.00	6.86
Flavonoid content (mg QE)	15.00	54.67

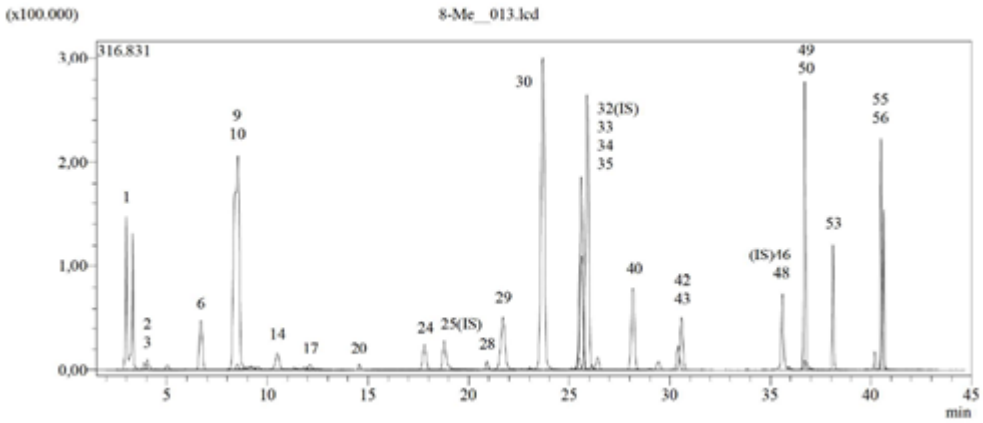
In our study, it was shown that *C. bursa-pastoris* water extracts have higher phenolic but lower flavonoid contents than methanol extracts. The ingredients of the polyphenolic molecules affect the antioxidant capabilities of these extracts. *C. bursa-pastoris* has been shown to have a considerable amount of phenolics. Fifty-three phenolic compounds were used as references, the LC-MS/MS analysis was employed to specify the chemical content and phenolic compounds of *C. bursa-pastoris* water and methanol extracts. Almost 23 compounds were measured in water extract and 25 compounds were determined in methanol extract of *C. bursa-pastoris* (Table 1). Table 2 indicates the mean levels of compounds according to the LC-MS/MS method. The extensive constituents identified in methanol extract of *C. bursa-pastoris* were quinic acid (22.629 mg/g), fumaric acid (11.029 mg/g), rutin (10.95 mg/g), cyranoside (9.463 mg/g), chlorogenic acid (6.072 mg/g), hesperidin (5.91 mg/g), isoquercitrin (5.364 mg/g), nicotiflorin (2.349 mg/g), cosmosiin (2.343 mg/g), protocatechuic acid (1.544 mg/g), astragalin (1.116 mg/g), p-coumaric acid (0.874 mg/g). Other detected compounds are aconitic acid, gallic acid, gentisic acid, 4-OH-benzoic acid, caffeic acid, salicylic acid, naringenin, hesperedin, luteolin, apigenin, chrysin, acacetin, and coumarin. The quinic acid was found in higher amount (22.629 mg/g) in water extract of *C. bursa-pastoris* aeral parts. But the remaining references of the LC-MS/MS analyze were not measured in either extract of *C. bursa-pastoris*.



A



B



C

Figure 1A. Chromatogram of all the standard phenolic compounds, **B.** Chromatogram of *C. bursa-pastoris* species water extract compounds. **C.** Chromatogram of *C. bursa-pastoris* species methanol extract

The investigation revealed that flavonoid derivative chemicals, which are main constituents of *C. bursa-pastoris* species, possess significant bioactivities. Antioxidant quinic acid is one of them; it has shown anticancer efficacy by causing cytotoxicity in breast cancer cells through apoptosis. Quinic acid has also demonstrated a strong affinity for selectins, which are angiogenesis factors that are more abundant in breast cancerous tissue [54]. Moreover, quinic acid, a polyphenol-rich substance, has been shown to have strong antihyperglycemic effects [55]. Naturally occurring, fumaric acid is an organic acid with a variety of qualities that may help with liver damage caused by cadmium, including neuroprotective and antioxidant effects [56].

Table 2. LC–MS/MS parameters of selected phenolic compounds in water and methanol extracts of *C. bursa-pastoris*

No	Analytes	Water extract (mg/g)	Methanol extract (mg/g)
1	Quinic acid	22.629	11.356
2	Fumaric aid	11.029	2.724
3	Aconitic acid	0.457	0.011
4	Gallic acid	0.018	N.D.
5	Epigallocatechin	N.D.	N.D.
6	Protocatechuic acid	0.898	1.544
7	Catechin	N.D.	N.D.
8	Gentisic acid	0.323	N.D.
9	Chlorogenic acid	3.211	6.072
10	Protocatechuic aldehyde	N.D.	0.102
11	Tannic acid	N.D.	N.D.
12	Epigallocatechin gallate	N.D.	N.D.
13	1,5-Dicaffeoylquinic acid	N.D.	N.D.
14	4-OH-Benzoic acid	N.D.	0.235
15	Epicatechin	N.D.	N.D.
16	Vanilic acid	N.D.	N.D.
17	Caffeic acid	0.485	0.117
18	Syringic acid	N.D.	N.D.
19	Vanillin	N.D.	N.D.
20	Syringic aldehyde	N.D.	0.05
21	Daidzin	N.D.	N.D.
22	Epicatechin gallate	N.D.	N.D.
23	Piceid	N.D.	N.D.
24	p-Coumaric acid	0.799	0.874
25	Ferulic acid-D3-IS	N.A.	N.A.
26	Ferulic acid	N.D.	N.D.
27	Sinapic acid	N.D.	N.D.
28	Coumarin	N.D.	0.019
29	Salicylic acid	0.088	0.292
30	Cyranoside	0.060	9.463
31	Miquelianin	N.D.	N.D.
32	Rutin	1.930	10.95
33	Isoquercitrin	0.783	5.364
34	Hesperidin	0.893	5.912
35	O-Coumaric acid	N.D.	N.D.
36	Genistin	N.D.	N.D.
37	Rosmarinic acid	N.D.	N.D.
38	Ellagic acid	N.D.	N.D.
39	Cosmosiin	0.016	2.343
40	Quercitrin	N.D.	N.D.
41	Astragalin	0.239	1.116
42	Nicotiflorin	0.378	2.349
43	Fisetin	N.D.	N.D.
44	Daidzein	N.D.	N.D.
45	Quercetin	N.D.	N.D.
46	Naringenin	0.003	0.027
47	Hesperetin	0.009	0.003
48	Luteolin	0.29	0.659
49	Genistein	N.D.	N.D.
50	Kaempferol	N.D.	N.D.
51	Apigenin	0.018	0.191
52	Amentoflavone	N.D.	N.D.
53	Chrysin	0.003	0.587
54	Acacetin	0.010	0.089

N.D.: Not Detected, N.A.: Not Applicable

A novel anti-diabetic drug including the plant flavonoids epicatechin, rutin, and catechin has been shown in several investigations to be efficacious. Furthermore, they are strong antioxidants and anti-inflammatory compounds. A novel, safe, multi-target anti-diabetic formulation that effectively manages diabetes and its complications will be created by combining them in the most efficient manner feasible through a mixture design experiment. Because of their strong antihyperglycemic qualities, rutin, catechin, and epicatechin work together to create a unique formulation that may be able to successfully compete with currently available drugs [57].

The effects of cyanaroside on inflammatory metabolic pathways, including glycolysis, diabetes, and cancer cells, as well as its anti-inflammatory activity and interactions with JAK/STAT3, NF-kB, and other pathways, have been all thoroughly described in the literature [58]. Regarding their antioxidant, antibacterial, and *in vitro* diabetes enzymes inhibitory properties, cyranoside and cosmosiin substances identified by LC-MS/MS in a variety of plant samples have been shown to have antidiabetic benefits [59]. In various antidiabetic targets, including α-amylase, α-glycosidase, and GLP1, docking's study indicates that cynaroside and cosmosiin are preferred molecules for hypoglycemic effects [60]. One well-known polyphenol, chlorogenic acid, has a number of significant biological qualities, including antioxidant, hepatoprotective, antiviral, anti-diabetic, and anti-hypertensive [19]. Through the genes linked to the metabolism of glucose and insulin signals, hesperidin may have an antidiabetic impact [61]. Isoquercitrin's α-glucosidase inhibitory impact was shown to be greater than acarbose's inhibitory effect in prior investigations [62]. In rats with multiinfarct dementia, nicotiflorin has protective benefits on lowering oxidative stress, cognitive impairment, and energy metabolism failure [63]. In diabetic rats, protocatechuic acid has antioxidant and antihyperlipidaemic properties; its effects are similar to those of the common medication, glibenclamide [64]. Antioxidant, neurological, and antidiabetic properties are among astragalin's pharmacological functions [65].

The reduction capability of a molecule can be a sign of possible antioxidant action of it. Antioxidant substances may give electrons to ROS, transforming them into more stable and non-reactive forms [34]. The antioxidant activity of *C. bursa-pastoris* may be due to its polyphenolic concentration. The reduction potentials of phenolic ingredients of *C. bursa-pastoris* were determined with three different reduction systems, together with Fe^{3+}, Cu^{2+} and Fe^{3+}-TPTZ reducing abilities. The radical removal features of the *C. bursa-pastoris* samples was evaluated by DMPD and ABTS radical scavenging methods. *C. bursa-pastoris* natural compounds may have reducing properties, thereby neutralizing oxidants and free radicals.

The direct reduction of $Fe^{3+}(CN^{-})_6$ to $Fe^{2+}(CN^{-})_6$ and the absorbance that results from the constitution of the Perl's Prussian blue complex after the addition of excess ferric ions (Fe^{3+}) were used to measure the ability of *C. bursa-pastoris* extracts to reduce Fe^{3+}. To appraise the reducing capacity of *C. bursa-pastoris* extracts, the ferric reducing antioxidant power Oyaizu method [33] was applied with a minor adjustment [66]. In this method, Fe^{3+} would be converted to Fe^{2+} in the existence plant samples as reductants [67]. The supplementation of ferric ions (Fe^{3+}) to compound causes $Fe_4[Fe(CN^{-})_6]_3$ complex creation, which bring about a peak absorbtion at 700 nm [68]. As abbreviated in Table 2 and Figure 3A, *C. bursa-pastoris* extract indicated a powerful Fe^{3+} reducing outline. Fe^{3+} reducing capability of 30 µg/mL amount of *C. bursa-pastoris* specimens and references orders: α-tocopherol (2.778±0.248, r^2:0.9999) > Trolox (2.334±0.167, r^2: 0,9997) > BHA (2.319±0.041, r^2:0.9629) > BHT (1.873±0.152, r^2: 0.9918) > *C. bursa-pastoris*-Methanol (0.381±0.007, r^2:0.9932 > *C. bursa-pastoris* species-Water (0.280±0.027, r^2:0.9906). Depending on how well the extracts of *C. bursa-pastoris* reduce antioxidants, the reaction colours change to different dgrees of blue and green colour.

The CUPRAC technique shows that the presence of antioxidants reduces the cupric neocuproine [Cu^{2+}-Nc] complex, which has the highest light absorption at 450 nm [69]. Cu^{2+} reducing capabilities of phenolic ingredients in *C. bursa-pastoris* samples are given in Table 3 and Figure 3B. It was shown that the Cu^{2+} reducing effect and the various phenolic component contents in *C. bursa-pastoris* extracts were strongly correlated. Nevertheless, phenolic compounds in *C. bursa-pastoris* extracts showed a substantial absorption of reducing power at 30 µg/mL. However, the following results were obtained regarding the extracts and standards of *C. bursa-pastoris's* capacity to reduce Cu^{2+} ions: BHT (2.865±0.038, r^2:0.9991) > BHA (2.849±0.020, r^2:0.99994) > Trolox (2.555±0.022, r^2: 0.9987) > α-Tocopherol (2.185±0.110, r^2:0.9986) > *C. bursa-pastoris*-Methanol 20 (0.372±0.023, r^2:0.9872) > *C. bursa-pastoris*-Water

(0.267±0.013, r^2:0.9925). For a range of antioxidant substances, the CUPRAC method is quick, stable, affordable, selective, and easy to use [70,71].

The FRAP test can be utilized to calculate the overall reduction potential of plant extracts or absolute antioxidant substances. The electron transfer mechanism that forms the basis of the FRAP test uses a ferric salt as an oxidant. Fe^{2+} may be detected spectrophotometrically because of its colorful interaction with TPTZ, At 593 nm, its absorption reaches its maximum [72]. This method is exclusively successful in specifying the reduction activities of biomolecules. The FRAP test uses a redox-linked colorimetric technique in which the antioxidants in the specimen are employed as reducing agents. Second, the FRAP test procedure is an extremely straightforward and standardizeable one [38]. To appreciate the efficiency of biological agents and pure chemical liquids in reducing ferric iron, the FRAP test was developed. Furthermore, it has been employed to specify the antioxidant potential of phenolic substances [73]. The assay's yellow test solution turns into a range of green and blue hues, attached to how strong the reducing agent is in the antioxidant substance.

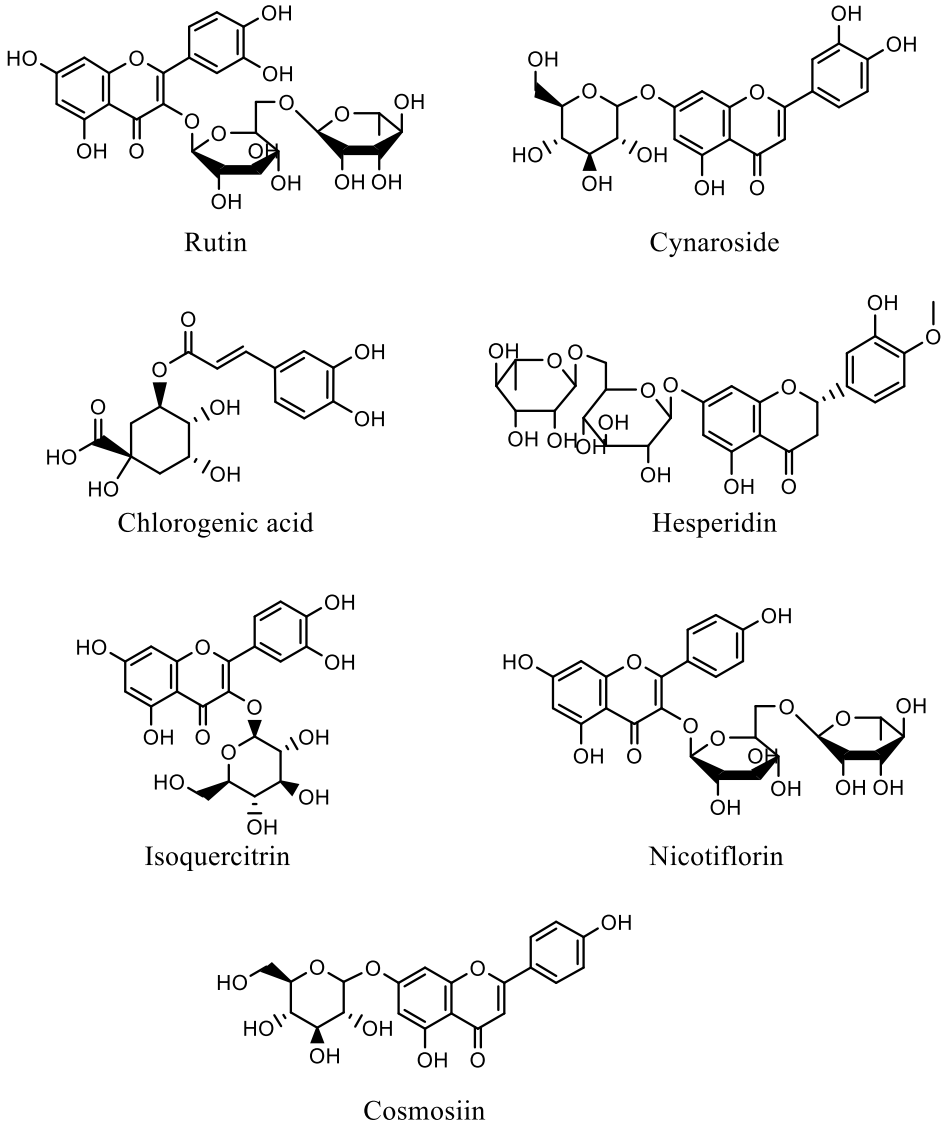


Figure 2. The ten phenolic compounds that are most prevalent in the *C. bursa-pastoris*

C. bursa-pastoris extracts and standards were evaluated for their reducing ability using the Fe^{3+}-TPTZ reduction test. Based on the data indicated in Table 3 and Figure 3C, the samples' reduction activities listed below: α-tocopherol (2.434±0.103, r^2:0.8714) > BHA (2.151±0.020, r^2:0.9367) > Trolox (2.108±0.026, r^2: 0.9291) > BHT (2.031±0.190, r^2: 0.9670) > *C. bursa-pastoris*-Methanol 20

(0.680±0.050, r^2: 0.9472) > *C. bursa-pastoris*-Water (0.622±0.016, r^2: 0.9727). These results were recorded with the 30 µg *C. bursa-pastoris* specimens.

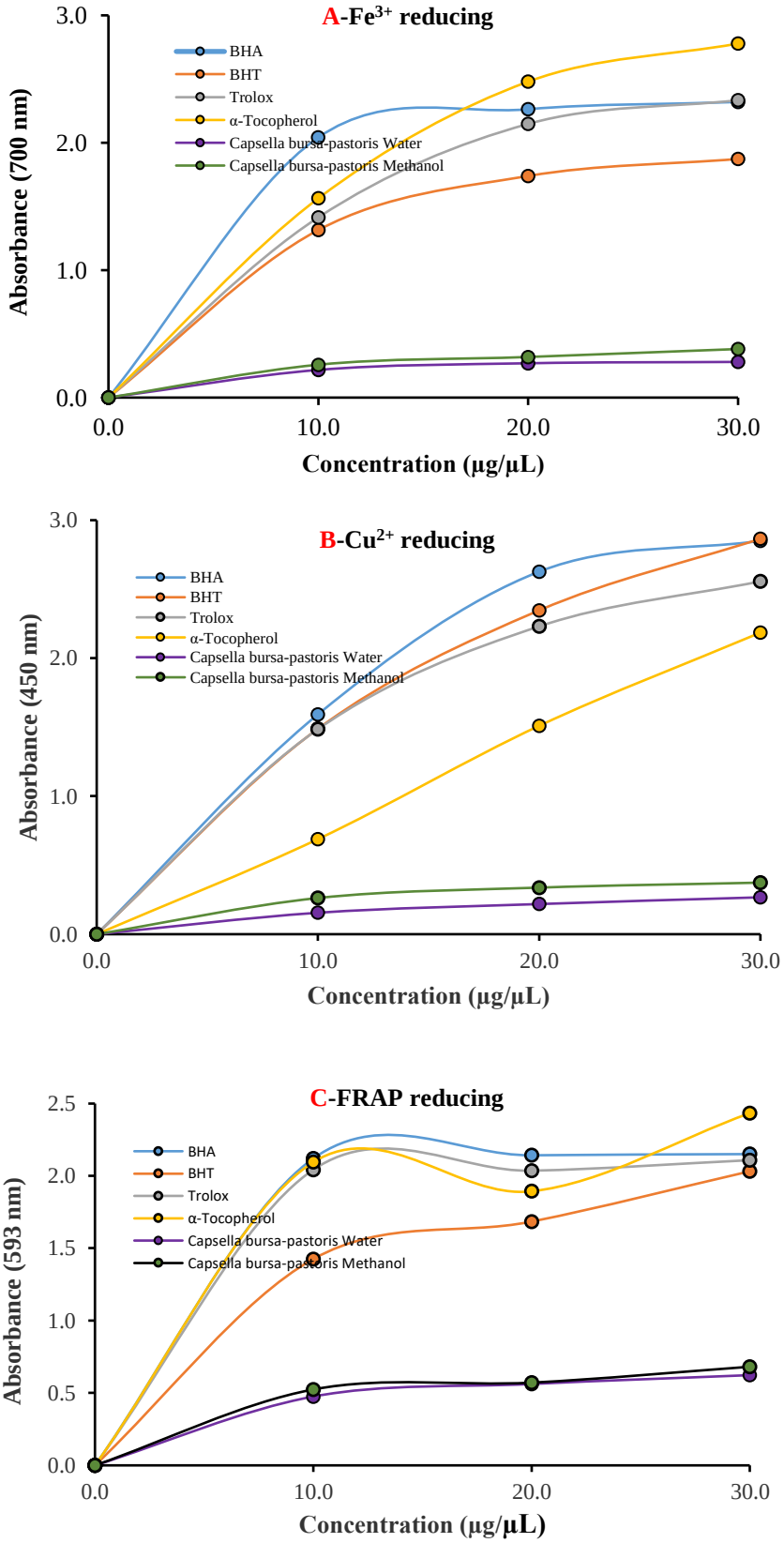


Figure 3. A. Fe^{3+} reducing assay, **B**. Cu^{2+}reducing assay, **C**. Fe^{3+}-TPTZ reducing abilities of *C. bursa-pastoris* and standards.

Table 3. Fe^{3+}, Cu^{2+} and Fe^{3+}-TPTZ ions reduction capabilities of *C. bursa-pastoris* species and positive controls at 30 μg/mL concentration.

Antioxidants	Fe^{3+} reducing		Cu^{2+} reducing		Fe^{3+}-TPTZ reducing	
	700 nm	**r^2**	**450 nm**	**r^2**	**593 nm**	**r^2**
BHA	2.319±0.041	0.9629	2.849±0.020	0.9994	2.151±0.020	0.9367
BHT	1.873±0.152	0.9918	2.865±0.038	0.9991	2.031±0.190	0.967
Trolox	2.334±0.167	0.9997	2.555±0.022	0.9987	2.108±0.026	0.9291
α-Tocopherol	2.778±0.248	0.9999	2.185±0.110	0.9986	2.434±0.103	0.8714
C. bursa-pastoris-Water	0.280±0.027	0.9906	0.267±0.013	0.9925	0.622±0.016	0.9727
C. bursa-pastoris-Methanol	0.381±0.007	0.9932	0.372±0.004	0.9872	0.680±0.050	0.9472

The $ABTS^{\cdot+}$ radical scavenging-based methods, a green blue chromogens is also simple to use, sensitive, fast, selective, and reproducible to evaluate antioxidative qualities of drinks, meals herbals [74]. ABTS radicals were occured in an $ABTS/K_2S_2O_8$ medium. Before the radical is exposed to possible antioxidants, it is first generated directly in a stable state via a decolorization process. The blue/green $ABTS^{\bullet+}$ chromophore is directly produced by the reaction of potassium persulfate with ABTS in the enhanced process for creating $ABTS^{\bullet+}$ that is detailed here [17]. Absolute substances, liquid mixes, and soft drinks can all have their total antioxidant capacity measured using a spectrophotometric approach based on the generation of the ABTS radical cation [75]. The ABTS radical scavenging potential of the *C. bursa-pastoris* herb ethanol and aqueaus extracts were found as ethanol extract EC_{50} = 0.09 mg/mL) > aqueaus extract (EC_{50} = 0.98 mg/mL) [9]. The ABTS test was used to examine the antioxidant capability of *C. bursa-pastoris*. The study's findings indicated that *C. bursa-pastoris*'s ABTS radical scavenging activity rose as its concentration increased, with an IC_{50} value of 61.6 μg/mL. About 80% of the ABTS free radical was scavenged at a quantity of 160 μg/mL [76].

C. bursa-pastoris extracts and standards IC_{50} values were given in the rank of 53.3 μg/mL for *C. bursa-pastoris* methanol extract, 49.5 μg/mL for *C. bursa-pastoris* water extract, 9.5 μg/mL for BHT, 8.9 μg/mL for α-tocopherol, 8.8 μg/mL for BHA, and 8.6 μg/mL for Trolox, (Table 4 and Figure 5A). The data indicated that *C. bursa-pastoris* as a natural antioxidant source has effective $ABTS^{\cdot+}$ scavenging capability. *C. bursa-pastoris* extracts samples showed a lower radical scavenging effect than synthetic antioxidants.

The IC_{50} values of DMPD radical scavenging activity of *C. bursa-pastoris* water extract and Trolox were 69.3 μg/mL for the water extract and 43.3 μg/mL for Trolox (Table 4). Some extractive fractions obtained from *C. bursa-pastoris* moiety, and the compounds had robust capacity to scavenge free radicals, and the presence of a number of compounds' reduction powers were on par with or greater than those of the positive control trolox. Oxidative damage to HT-22 cells can be prevented by the ethyl acetate fraction, n-buthanol fraction, and a number of specific chemicals [77]. The herbal extract of *C. bursa-pastoris* has a nephroprotective capacity versus nephropathy, according to the biochemical results and kidney histological analysis [3].

Table 4. The IC_{50} (μg/mL) values of *C. bursa-pastoris* extracts and standards for $ABTS^{\bullet+}$ and $DMPD^{\bullet+}$ scavenging and for Fe^{2+} chelating abilities

Antioxidants	$ABTS^{\bullet+}$ scavenging		DMPD scavenging		Fe^{2+} chelating	
	IC_{50}	**r^2**	**IC_{50}**	**r^2**	**IC_{50}**	**r^2**
BHA	8.8	0.9959	-	-	-	-
BHT	9.5	0.9999	-	-	-	-
Trolox	8.6	0.9349	43.3	0.9338	-	-
α-Tocopherol	8.9	0.9999	-	-	-	-
Ascorbic acid	-	-	-	-	20.4	0.8445
EDTA	-	-	-	-	3.8	0.9474
C. bursa-pastoris-Water	49.5	0.9928	69.3	0.9963	21.0	0.9951
C. bursa-pastoris-Methanol	53.3	0.9959	-	-	19.3	0.9703

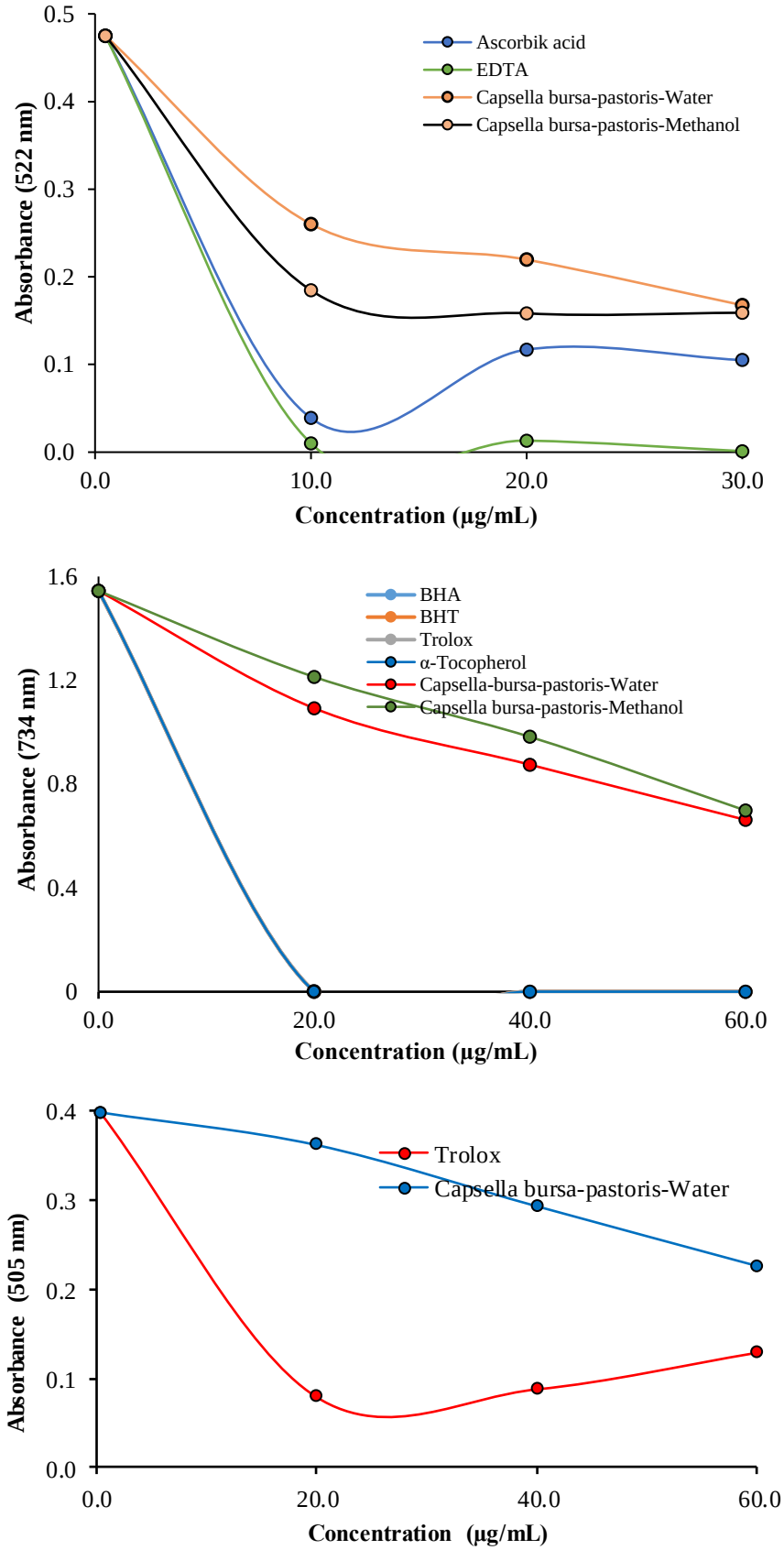


Figure 5. Metal chelating and radical scavenging effects of *C. bursa-pastoris* and positive controls. **A.** Fe^{2+} chelating, **B**. $ABTS^{\bullet+}$ scavenging ability. **C.** $DMPD^{\bullet+}$ scavenging ability.

The IC_{50} Fe^{2+} chelation values of *C. bursa-pastoris* species specimens were found to be in the rank of 19.3 to 21.0 µg/mL. Also, the IC_{50} values for Fe^{2+} chelation was measured as 20.4 µg/mL for ascorbic acid and 3.8 µg/mL for EDTA (Table 5 and Figure 4).

Essential glycoside hydrolases for the appropriate digestion of carbohydrates are α-amylase and α-glycosidase enzymes. The cells lining the colon contain both enzymes, which hydrolyze polysaccharides into absorbable monosaccharide units. To accomplish regulating body weight and control blood glucose levels, certain inhibitors can stop these enzymes from functioning. Plant-based foods contain inhibitory chemicals in a comparatively harmless manner [78]. The inhibition of α-amylase aids in the prevention and management of hyperglycemia because it is significant for the digestion of dietary carbohydrates. The inhibition of α-amylase by natural materials, such plant extracts, undergo several investigations nowadays [79]. In another research, the ethanol extract of *C. bursa-pastoris* shown considerable in vitro inhibition of α-amylase, which causes postprandial hyperglycemia in diabetic rats, as well as promising effects in reducing blood glucose and lipid levels in rats with STZ-induced diabetes. The usual medication acarbose inhibited α-amylase (IC_{50} =0.46 ± 0.02 mg/mL), but the ethanol extract of *C. bursa-pastoris* inhibited α-amylase (IC_{50} = 4.28 ± 0.81 mg/ mL) [1]. α-Amylase inhibitory effect of methanol extract of *C. bursa-pastoris* were determined, and the results were shown in Table 6. For α-amylase, *C. bursa-pastoris* species water and methanol extracts had IC_{50} values in the rank of 122.4 µg/mL to 245.9 µg/mL. The inhibiton values of water extract of *C. bursa-pastoris* was higher than *C. bursa-pastoris* species methanol extract. In the literature it was the first measurement of *C. bursa-pastoris* species inhibiton against α-amylase enzyme.

The leading neurological condition and the main contributor to cognitive impairments in elders is AD. The most prominent biochemical change associated with AD is decreased quantity of AChE in the brain [80-82]. Treatment for AD involves the use of AChE inhibitory medications. Nevertheless, these drugs have a few unfavorable advers consequences. Consequently, there is an immediate requirement to investigate and employ more powerful antioxidants and AChE agents [83-85]. Additionally, AChE inhibitory properties were found to be mostly associated with aromatic chemicals and, to a smaller degree, aliphatic compounds [86-88]. Although they are utilized to treat AD, acetylcholinesterase inhibitors only have short-term results. Cholinesterase inhibitors are abundant in medicinal plants. Cholinergic enzyme inhibition in medicinal plants is mostly caused by phenolic compounds [89-91]. The *C. bursa-pastoris* species methanol and water extracts inhibition level was lower but not to far to the tacrine standard, as a well-known AChE enzyme inhibitor. Table 6 compiles the IC_{50} levels of *C. bursa-pastoris* species water and methanol extracts for enzyme inhibition. The IC_{50} value for *C. bursa-pastoris* species water and methanol extract against AChE enzyme inhibiton found between 19.0 to 20.9 µg/mL compared with Tacrine, in Table 6. Tacrine's IC_{50} was 5.97 µg/mL, which was utilized as a control for AChe enzyme inhibition.

The water and ethanol extract of *C. bursa-pastoris* indicated the lower inhibition contrary to AChE enzyme with IC_{50} level of > 200 µg/mL than the same plant's water and methanol extracts results in this study but the hexane extract of *C. bursa-pastoris* indicated the strongest inhibition towards AChE enzyme with IC_{50} level of 7.24 µg/mL in that study [90]. IC_{50} of antioxidant experiment of ABTS in the *C. bursa-pastoris* which is used as food by the Gaddis-a tribal tribe in the Western Himalayas, was found 5.033 µg/ml [92]. AChE inhibition power of *C. bursa-pastoris* methanol extracs enzyme inhibition value was 909.44 µg/mL and methanol/water extracts enzyme inhibition value was 3579.41 µg/mL [92]. In this study, it was shown in Table 6 that *C. bursa-pastoris* extracts effectively inhibited the AChE enzyme.

Table 6. The inhibition values of *C. bursa-pastoris* species methanol and water extracts against acetylcholinesterase (AChE) and α-amylase enzymes.

Antioxidants	**IC_{50} (µg/mL)**			
	AChE	**r^2**	**α-Amylase**	**r^2**
C. bursa-pastoris-Water	20.9	0.9611	168.6	0.9154
C. bursa-pastoris-Methanol	19.0	0.9364	238.6	0.7990
Tacrine*	5.97	0.9706		
Acarbose**	-	-		

*Tacrine (TAC) is a standard for AChE inhibition

**Acarbose (ACR) is a standard for α-amylase inhibition

4. Conclusion

C. bursa-pastoris has bioactive content as phenolics and flavonoids; *C. bursa-pastoris* plants are known and used as medicinal plants in herbalists to eliminate various health problems and which we think have the power to contribute to human health. *C. bursa-pastoris* ingredients were shown to have antioxidant activity and inhibit AChE and α-amylase enzymes. *C. bursa-pastoris* plant is an important plant with its enzyme inhibitory, reducing and radical scavenging properties, which is a natural chemical that can be used to treat AD and severe, common T2DM, with the phenolic and flavonoids it contains, and can also be used in research in the food and pharmaceutical industry. Since the usage of artificial antioxidants has been forbidden in recent years, these plant extracts can be utilized in the context of alternative studies. According to the LC-MS/MS the major constituion detected in *C. bursa-pastoris* methanol extracts were, quinic acid, fumaric acid, rutin, cyranoside, chlorogenic acid, hesperidin, ısoquercitrin, nicotiflorin and cosmosiin. Therefore, the methanol and water extracts of *C. bursa-pastoris* had high antioxidant activity, reducing power, phenolic contents, and inhibited AChE and α-amylase. Because of the abundance of phenolics and flavonoids it contains, *C. bursa-pastoris* extracts have antioxidant, reducing, and radical scavenging properties. Both extracts are acceptable for use as a natural product in the food and pharmaceutical industries as well as in the remedy of prevalent diseases, T2DM, AD, neurodegenerative, hormonal, and metabolic disorders. When clinical pharmacological investigations support the use of *C. bursa-pastoris* extracts for pharmacological motivation in people with the conditions, it is expected to produce positive outcomes in treatment.

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