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Unraveling the chemical profile, antioxidant, enzyme inhibitory, cytotoxic potential of different extracts from *Astragalus caraganae*

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Abstract

Six extracts (water, ethanol, ethanol-water, ethyl acetate, dichloromethane, and *n*-hexane) of *Astragalus caraganae* were studied for their biological activities and bioactive contents. Based on high-performance liquid chromatography-mass spectrometry (HPLC-MS), the ethanol-water extract yielded the highest total bioactive content (4242.90 µg g^{-1}), followed by the ethanol and water extracts (3721.24 and 3661.37 µg g^{-1}, respectively), while the least total bioactive content was yielded by the hexane extract, followed by the dichloromethane and ethyl acetate extracts (47.44, 274.68, and 688.89 µg g^{-1}, respectively). Rutin, *p*-coumaric, chlorogenic, isoquercitrin, and delphindin-3,5-diglucoside were among the major components. Unlike the dichloromethane extracts, all the other extracts showed radical scavenging ability in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (8.73–52.11 mg Trolox equivalent [TE]/g), while all extracts displayed scavenging property in the 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging assay (16.18–282.74 mg TE/g). The extracts showed antiacetylcholinesterase (1.27–2.73 mg galantamine equivalent [GALAE]/g), antibutyrylcholinesterase (0.20–5.57 mg GALAE/g) and antityrosinase (9.37–63.56 mg kojic acid equivalent [KAE]/g) effects. The molecular mechanism of the H_2O_2-induced oxidative stress pathway was aimed to be elucidated by applying ethanol, ethanol/water and water extracts at 200 µg/mL concentration to human dermal cells (HDFs). *A. caraganae* in HDF cells had neither a cytotoxic nor genotoxic effect but could have a cytostatic effect in increasing concentrations. The findings have allowed a better insight into the pharmacological potential of the plant, with respect to their chemical entities and bioactive contents, as well as extraction solvents and their polarity.

KEYWORDS
antioxidant, *Astragalus caraganae*, bioactive compounds, extraction, solvents

1 | INTRODUCTION

Astragalus (Fabaceae) is regarded to be the largest genus of vascular plants with an estimation of 2500–3000 species,[1] highly valued for their medicinal uses. For centuries, a wide range of *Astragalus* species have been used in the traditional medicine of different countries such as Pakistan, Iran, India, Korea, and China.[2,3]

The *Astragalus* genus has been extensively studied, mainly due to the presence of three major groups of bioactive compounds, namely flavonoids, polysaccharides, and saponins. Furthermore, other compounds, including sesquiterpene-flavonolic complexes, lignans, sterols, coumarins, and phenolic acids, have also been reported to possess biological activities. Among these, flavonoids constitute the largest group of polyphenolic compounds present in *Astragalus* species.[4] Besides, *Astragalus* polysaccharide, another important natural active component in some species, has been found to exert a panoply of pharmacological properties and hence has huge potential in the development of drugs for treating different diseases.[5] Indeed, several works have elaborated on the phytochemistry of this genus, establishing their importance in drug discovery.[6–10]

In addition to its tonic and diuretic effects, *Astragalus* is commonly used in traditional medicine for treating various conditions including diabetes, leukemia, nephritis, and uterine cancer. It is also used to address kidney and urinary complications, liver and digestion problems, female reproductive issues, skin disorders, and ailments related to the cardiovascular, immune, lymphatic, nervous, and respiratory systems, among others.[11] From a scientific perspective also, pharmacological research demonstrates the crude extracts and components of some species of *Astragalus* to enhance telomerase activity, to show antioxidant, anti-inflammatory, anticancer, immunoregulatory, hepatoprotective, hypolipidemic, antihyperglycemic, expectorant, and diuretic effects, as well as, cardioprotective, and antiviral activities.[12–17]

While much information is available on the pharmacological actions and benefits of some *Astragalus* species,[18] others have remained uncovered and hence warrant more attention. Hence, this study attempts to investigate the chemical profiling and biological activities of different extracts of *Astragalus caraganae*, with in vitro, cytotoxic and in silico perspectives.

2 | RESULTS AND DISCUSSION

2.1 | Chemical characterization

Nearly 20% of known plants have been employed in pharmaceutical industry, impacting positively the healthcare system by assisting in the treatment of cancer and other diseases. Plants produce a vast number of miscellaneous bioactive compounds. Plants containing useful phytochemicals may supplement the requirements of the human body by acting as natural antioxidants.[19] Plants are a vital source of numerous active ingredients with diverse pharmacological effects that are widely utilized in the pharmaceutical industry. These compounds have potential in the treatment of chronic-degenerative diseases like diabetes and cancer, among others.[20]

Phenolic compounds are the most stable and important secondary metabolites found in the plant kingdom, contributing to the remarkable diversity of compounds that plants are able to produce.[21] Phenolic compounds are produced through the shikimic acid and pentose phosphate pathways of plants during phenylpropanoid metabolism. They consist of benzene rings with one or more hydroxyl groups and range from simple phenolic molecules to highly polymerized compounds.[22] Phenolic compounds have demonstrated several health benefits, including anticarcinogenic, cardioprotective, immune-boosting, antibacterial, antiviral, and antifungal properties. Moreover, they have been reported to provide skin protection against UV radiation.[23]

The extraction and purification of phytochemicals and antioxidant compounds from plant materials can be influenced by various factors, such as temperature, time, and solvent concentration and polarity. Due to the diverse chemical nature of phytochemicals, a range of solvents with different polarities can be used for their extraction, as no single solvent can effectively extract all phytochemicals and antioxidant compounds present in plant materials.[24] In this study, water, ethanol, ethanol-water, ethyl acetate, dichloromethane and *n*-hexane, possessing different degrees of polarity, were used as solvents.

In the present study, the quantitative total phenolic and flavonoid contents of the extracts were determined using spectrophotometric-colorimetric assays. All extracts possessed total phenolic and flavonoid contents in varying amounts. For instance, the extracts yielded TPC ranging from 22.80 to 73.69 mg GAE/g and TFC ranging from 0.65 to 22.12 mg RE/g. The more polar extracts (water, ethanol, ethanol-water, ethyl acetate) were found to yield higher TPC (55.61–73.69 mg GAE/g) compared to the less polar ones (*n*-hexane and dichloromethane) (22.80 and 22.91 mg GAE/g, respectively). A similar tendency was noted for extracts' TFC as well, whereby the more polar solvent extracts yielded higher TFC compared to the least polar ones (Table 1).

TABLE 1 Extraction yields, total phenolic, and flavonoid content of the tested extracts.

Extracts	Extraction yields (%)	Total phenolic content (mg GAE/g)	Total flavanoid content (mg RE/g)
n-Hexane	0.91	22.80 ± 0.25^{d}	0.65 ± 0.09^{f}
Ethyl acetate	2.90	73.69 ± 1.53^{a}	4.66 ± 0.58^{d}
Dichloromethane	1.36	22.91 ± 1.13^{d}	2.88 ± 0.19^{e}
Ethanol	10.82	68.59 ± 1.35^{b}	11.95 ± 0.16^{c}
Ethanol/water	8.96	55.61 ± 0.54^{c}	22.12 ± 0.14^{a}
Water	11.72	65.57 ± 1.22^{b}	18.89 ± 0.05^{b}

Note: Values are reported as mean ± SD of three parallel measurements. Different letters indicate significant differences between the tested extracts ($p < 0.05$).

Abbreviations: GAE, gallic acid equivalents; RE, rutin equivalents.

Further phytochemical analysis was conducted using high performance liquid chromatography-mass spectrometry (HPLC-MS) technique. The polar solvent extracts were found to yield higher total bioactive content compared to the less polar ones. For instance, ethanol-water extract was revealed to have the highest total bioactive content (4242.90 $\mu g\,g^{-1}$), followed by the ethanol and water extract (3721.24 and 3661.37 $\mu g\,g^{-1}$, respectively). On the other hand, the least total bioactive content was yielded by hexane extract, followed by dichloromethane and ethyl acetate extracts (47.44, 274.68, and 688.89 $\mu g\,g^{-1}$, respectively) (Table 2). Moreover, some phytochemicals were found to be commonly present in all extracts although they varied in quantity, such as chlorogenic acid (2.98–243.21 $\mu g\,g^{-1}$), 4-hydroxy benzoic acid (11.07–87.04 $\mu g\,g^{-1}$), *p*-coumaric acid (7.04–505.66 $\mu g\,g^{-1}$), ferulic acid (0.66–25.54 $\mu g\,g^{-1}$), rutin (4.09–2256.99 $\mu g\,g^{-1}$), isoquercitrin (0.44–277.61 $\mu g\,g^{-1}$), delphindin-3,5-diglucoside (0.19–288.43 $\mu g\,g^{-1}$), kaempferol-3-glucoside (0.43–88.24 $\mu g\,g^{-1}$), isorhamnetin (0.05–8.69 $\mu g\,g^{-1}$), hyperoside (0.53–270.79 $\mu g\,g^{-1}$), hesperidin (2.47–12.34 $\mu g\,g^{-1}$), and *trans*-cinnamic acid (17.26–126.38 $\mu g\,g^{-1}$). Interestingly, the components that contributed largely to the total bioactive content of the polar extracts were rutin, *p*-coumaric, chlorogenic, isoquercitrin, and delphindin-3,5-diglucoside (Table 2). Rutin has been shown to have an extensive array of healing applications due to its numerous properties including antioxidant activities. Several mechanisms have been described to be accountable for its antioxidant activities in both in vitro and in vivo models.[25] Other phenolic acids such as *p*-coumaric and chlorogenic acids, were also prevalent in the polar extracts. Phenolic acids are a subclass of plant phenolics characterized by a phenol moiety and resonance-stabilized structure that facilitates hydrogen atom donation, resulting in antioxidant activity through radical scavenging mechanisms. Additionally, they possess other mechanisms, such as electron donation-based radical quenching and singlet oxygen quenching, contributing to their antioxidant properties. Apart from their antioxidant activity, phenolic acids are found abundantly in plants and are known to possess several other health-protective effects, such as anti-inflammatory, antimicrobial, anticancer, and antimutagenic activities.[26]

2.2 | Antioxidant effects

In radical scavenging (2,2-diphenyl-1-picrylhydrazyl [DPPH] and 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) [ABTS]) and reducing (cupric reducing antioxidant capacity [CUPRAC] and ferric reducing antioxidant power [FRAP]) assays, more polar extracts were found to exert better antioxidant properties compared to less polar extracts. For instance, in the DPPH and ABTS assays, the more polar extracts such as water, ethanol-water, ethanol, and ethyl acetate showed scavenging abilities in the range of 31.07–52.11 mg TE/g and 249.08–291.64 mg TE/g, respectively. The dichloromethane extract did not show scavenging properties in DPPH assay. A similar trend was noted for the more polar extracts in the reducing assays CUPRAC and FRAP (265.84–503.81 mg TE/g and 80.79–128.31 mg TE/g, respectively). Other antioxidant assays were carried out. For instance, the extracts' total antioxidant capacity ranged from 1.45 to 2.68 mmol TE/g in phosphomolybdenum assay, while their metal chelating power ranged from 9.77 to 47.48 mg EDTAE/g. Ethyl acetate and water extracts showed the highest activity in phosphomolybdenum and metal chelating assay, respectively (Table 3). The relatively polarity-dependent increase in scavenging activity and reducing properties noted herein suggests that there could be extraction of strong antioxidant compounds in polar solvents compared to less or nonpolar ones, as previously reported.[24]

2.3 | Enzyme inhibitory effects

Postprandial hyperglycemia is a hallmark of diabetes and its management is a crucial aspect of treatment.[27] Carbohydrates are broken down into glucose by the enzymes α-amylase and α-glucosidase. Inhibitors of these enzymes can help delay the rise in blood glucose levels after a meal in diabetic patients. While effective inhibitors such as acarbose, voglibose, and miglitol are available, their gastrointestinal side effects make them unsuitable for long-term treatment.[28] In this regard, extracts from medicinal plants and their phytochemicals have been found to play an exceptional role in the management of diabetes, while having lesser side effects.[29–31]

In the current work, all extracts displayed antidiabetic effect through inhibition of amylase and glucosidase (0.04–0.56 and 0.93–1.09 mmol ACAE/g, respectively) (Table 4). While the less polar extracts showed lower amylase inhibition compared to the polar ones, the polar extracts on the other hand, displayed slightly higher antiglucosidase effect than the less polar ones.

Hyperpigmentation is a common dermatological issue that can be challenging to treat, but cosmeceuticals are frequently used for this purpose. Skin-lightening agents are often employed to selectively target hyperplastic melanocytes and inhibit key steps in melanin synthesis, with the goal of regulating melanin production.[32] Tyrosinase, a copper-containing enzyme that catalyzes two rate-limiting reactions in melanogenesis, is a promising target for inhibition. In addition to its relevance for treating hyperpigmentation, tyrosinase inhibition may also be of interest in the context of neuromelanin formation in the human brain, which has been linked to neurodegeneration in Parkinson's disease. It's worth noting that tyrosinase is also responsible for unwanted browning in fruits and vegetables.[33] Hence, tyrosinase inhibitors with high effectiveness and fewer adverse effects, have enormous demand in cosmetic, pharmaceutical, and food industries.[34] In this context, recent tendencies in the discovery of tyrosinase inhibitors derived from plants, have provided a rationale for the continued research on natural tyrosinase inhibitors.[35,36]

In the present study, while all extracts showed tyrosinase inhibition (9.37–63.56 mg KAE/g), the water extract did not show any tyrosinase inhibitory activity. Ethanol and ethanol-water extracts

TABLE 2 Content (μg g^{-1} of dried extract) of bioactive compounds in *Astragalus caraganae* extracts.

No.	Compounds	Hexane	EA	DCM	Ethanol	EtOH/H_2O	Infusion
1	Gallic acid	n.d.	1.52	0.00	5.54	37.27	26.09
2	Neochlorogenic acid	n.d.	0.77	0.00	15.19	44.02	60.13
3	Catechin	n.d.	n.d.	n.d.	0.89	4.78	0.71
4	Procyanidin B2	n.d.	n.d.	n.d.	3.33	26.64	9.16
5	Chlorogenic acid	2.98	27.41	7.24	103.70	196.82	243.21
6	4-Hydroxybenzoic acid	11.07	32.20	24.09	52.84	66.50	87.04
7	Epicatechin	n.d.	n.d.	n.d.	0.42	17.05	7.90
8	3-Hydroxybenzoic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	Caffeic acid	n.d.	4.75	n.d.	16.31	15.57	17.50
10	Vanillic acid	n.d.	2.14	n.d.	7.48	11.76	10.69
11	Syringic acid	n.d.	3.51	5.85	7.03	9.84	11.24
12	Procyanidin A2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	*p*-Coumaric acid	7.04	252.81	63.50	471.29	415.56	505.66
14	Ferulic acid	0.66	19.88	25.54	22.75	20.99	17.45
15	3,5-Dicaffeoylquinic acid	n.d.	4.50	0.35	24.45	26.56	9.56
16	Rutin	4.21	92.82	4.09	1918.32	2256.99	1953.18
17	Isoquercitrin	0.55	53.40	0.44	269.81	277.61	186.70
18	Delphindin-3,5-diglucoside	0.19	55.80	1.19	279.04	288.43	187.41
19	Phloridzin	n.d.	0.11	0.06	0.46	0.33	0.12
20	Quercitrin	n.d.	7.23	0.68	44.35	46.27	31.82
21	Myricetin	n.d.	0.28	0.00	1.00	5.69	0.28
22	Naringin	n.d.	0.00	0.00	0.00	0.00	0.00
23	Kaempferol-3-glucoside	0.43	16.12	1.09	72.74	70.80	88.24
24	Ellagic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
25	Quercetin	n.d.	4.05	0.38	36.09	67.58	22.19
26	Phloretin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
27	Isorhamnetin	0.05	0.92	2.39	5.09	8.69	3.29
28	Delphindin-3-galactoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
29	Cyanidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
30	Petunidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
31	Pelargonidin-3-rutinoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
32	Pelargonidin-3-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
33	Malvidin-3-galactoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
34	Hyperoside	0.53	61.31	0.61	270.79	252.26	117.72
35	Hesperidin	2.47	12.34	10.80	3.95	4.44	5.18
36	Kaempferol	n.d.	2.15	n.d.	35.40	27.19	14.00
37	*trans*-Cinnamic acid	17.26	32.86	126.38	52.98	43.26	44.84
Total content		47.44	688.89	274.68	3721.24	4242.90	3661.37

TABLE 3 Antioxidant properties of the tested extracts.

Extracts	DPPH (mg TE/g)	ABTS (mg TE/g)	CUPRAC (mg TE/g)	FRAP (mg TE/g)	PBD (mmol TE/g)	MCA (mg EDTAE/g)
n-Hexane	8.73 ± 0.56^{d}	26.32 ± 0.42^{e}	57.33 ± 0.77^{f}	17.74 ± 0.55^{d}	1.53 ± 0.09^{c}	40.34 ± 3.31^{b}
Ethyl acetate	52.11 ± 1.01^{a}	291.64 ± 1.57^{a}	503.81 ± 8.08^{a}	128.31 ± 5.40^{a}	2.68 ± 0.17^{a}	25.54 ± 1.94^{c}
Dichloromethane	n.a.	16.18 ± 0.68^{f}	74.15 ± 0.62^{e}	23.10 ± 0.50^{d}	1.45 ± 0.06^{c}	43.61 ± 3.50^{ab}
Ethanol	31.07 ± 1.19^{c}	282.74 ± 4.75^{b}	340.70 ± 9.27^{b}	93.92 ± 2.99^{b}	2.09 ± 0.01^{b}	9.77 ± 1.12^{e}
Ethanol/water	37.51 ± 1.24^{b}	249.08 ± 1.58^{d}	265.84 ± 7.56^{d}	83.43 ± 1.93^{c}	1.45 ± 0.02^{c}	16.12 ± 1.02^{d}
Water	31.97 ± 2.52^{c}	264.92 ± 2.48^{c}	286.26 ± 3.41^{c}	80.79 ± 0.23^{c}	1.53 ± 0.01^{c}	47.48 ± 1.25^{a}

Note: Values are reported as mean ± SD of three parallel measurements. Different letters indicate significant differences between the tested extracts ($p < 0.05$)

Abbreviations: ABTS, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); CUPRAC, cupric reducing antioxidant capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTAE, EDTA equivalent; FRAP, ferric reducing antioxidant power; MCA, metal chelating activity; na, not active; PBD, phosphomolybdenum; TE, trolox equivalent.

TABLE 4 Enzyme inhibitory effects of the tested extracts.

Extracts	AChE (mg GALAE/g)	BChE (mg GALAE/g)	Tyrosinase (mg KAE/g)	Amylase (mmol ACAE/g)	Glucosidase (mmol ACAE/g)
n-Hexane	2.30 ± 0.07^{b}	1.39 ± 0.07^{d}	14.70 ± 1.77^{d}	0.56 ± 0.02^{a}	0.93 ± 0.01^{c}
Ethyl acetate	2.73 ± 0.02^{a}	3.71 ± 0.36^{b}	9.37 ± 0.92^{d}	0.33 ± 0.01^{c}	1.02 ± 0.02^{b}
Dichloromethane	2.23 ± 0.09^{b}	2.29 ± 0.26^{c}	21.29 ± 4.27^{c}	0.49 ± 0.01^{b}	0.97 ± 0.03^{c}
Ethanol	n.a.	5.57 ± 0.07^{a}	63.56 ± 1.19^{a}	0.28 ± 0.01^{d}	1.07 ± 0.01^{ab}
Ethanol/water	2.72 ± 0.01^{a}	0.20 ± 0.05^{e}	56.44 ± 0.99^{b}	0.25 ± 0.01^{e}	1.09 ± 0.01^{a}
Water	1.27 ± 0.09^{c}	n.a.	n.a.	0.04 ± 0.01^{f}	1.09 ± 0.01^{a}

Note: Values are reported as mean ± SD of three parallel measurements. Different letters indicate significant differences between the tested extracts ($p < 0.05$).

Abbreviations: ACAE, acarbose equivalent; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; GALAE, galantamine equivalent; KAE, kojic acid equivalent; na, not active.

were found to display higher antityrosinase activity as opposed to the other extracts.

Acetylcholinesterase (AChE) is the primary enzyme that regulates the level of acetylcholine in a healthy brain, with butyrylcholinesterase (BChE) playing a minor role. However, in individuals with Alzheimer's disease, the activity of AChE decreases and that of BChE increases, resulting in a shift in the BChE to AChE ratio from 0.6 to as high as 11 in affected cortical areas. As such, inhibition of cholinesterase enzymes is an effective therapeutic approach for treating Alzheimer's disease symptoms.[37] Several studies have demonstrated the potential of phytocompounds and extracts from medicinal plants to exhibit AChE inhibitory activities that may prove beneficial in the treatment of Alzheimer's disease.[38–40]

In this study, all extracts except the ethanolic extract, showed anti-AChE potential (1.27–2.73 mg GALAE/g). The water extract showed the least anti-AChE activity. On the other hand, while the water extract did not show any inhibitory effect on BChE, the other extracts demonstrated anti-BChE ranging from 0.20 to 5.57 mg GALAE/g. The ethanol and ethanol-water extracts showed the highest anti-BChE and anti-AChE activity, respectively (Table 4).

Other members of the *Astragalus* genus have also been studied for their various pharmacological potentials, as screened for *A. caraganae* herein. For instance, in the study of Sarikurkcu, Sahinler, and Tepe,[41] the methanolic extracts of *Astragalus gymnolobu*, *Astragalus leporinus* var. *hirsutus*, and *Astragalus onobrychis* were tested for their phytochemical composition, antioxidant, tyrosinase, and α-amylase inhibitory effects. A strong correlation was noted between the phytochemical compositions of the extracts and their antioxidant activities. Besides this, the chloroform, ethyl acetate and *n*-butanol extracts of hydro-ethanolic extracts *Astragalus armatus* Willd. subsp. *numidicus* (Fabaceae) pods were assessed for their antioxidant properties, whereby the ethyl acetate extract exhibited the highest antioxidant activity in DPPH, ABTS, and CUPRAC assays. While the ethyl acetate extract afforded a flavonoid, the *n*-butanol extract gave four flavonoids, a cyclitol and a cycloartane-type saponin.[42] Other *Astragalus* species, notably *Astragalus campylosema* and *Astragalus hirsutus*, have been evaluated based on their biological, toxicological properties, and chemical profile.[43]

2.4 | Comparison of the effects of *Astragalus* extracts on advanced glycation end (AGE) inhibition

First, promising extracts with high phytochemical content and high antioxidant capacity were used in in vitro studies. Ethanol, ethanol/water, and water extracts, which have the richest content in total content analysis, as well as individual contents, were used in in vitro experiments. The molecular mechanism of the amelioration of cellular toxicity and other metabolic damage after the application of H_2O_2, one of the free radical phenomena, to commercially purchased HDF cells with *A. caraganae* extracts were elucidated, and thus, an attempt has been made to respond to the gaps in the literature. The molecular mechanism of the amelioration of cellular toxicity and other metabolic damage by *A. caraganae* extracts after applying H_2O_2, which is one of the free radical phenomena, to commercially purchased HDF cells has been elucidated and thus, it has been tried to answer the gaps in the literature.

AGE formation, which is known to play a role in the prognosis of many diseases, cellular DNA damage and development of genotoxicity, is basically a Maillard reaction.[44] AGE occurs as a result of reactions in the body between the carbonyl group of reducing sugars and an amino group of proteins, peptides, amino acids, and nucleic acids.[44] It has been determined that AGE has an active role especially in the activation of ROS-induced molecular pathways, and as a result of this, the nuclear integrity is disrupted, the DNA structure is damaged, and it has a mutagenic effect.[45–47] In line with this information, the effect of ethanol, ethanol/water and water extracts obtained from the aerial parts of *A. caraganae* at 0.5 and 1 mg/mL concentrations on AGE inhibition is presented in Figure 1. AGE experiments were performed at three independent time points and the results were analyzed in GraphPad Prism 8.6. As a result of the analysis, 62% inhibition was observed at 0.5 mg/mL of EtOH extract, while the inhibition activity at 1 mg/mL was 80% ($p \leq 0.01$ in Figure 1a). These values were found to be 70% and 85.3% in increasing concentrations of EtOH/water extract, respectively (**$p \leq 0.01$). In 0.5 mg/mL of water extract, the inhibition value is 58%, while at 1 mg/mL this value is 75% ($p \leq 0.01$ in Figure 1a). In Figure 1b, it was observed that the inhibitory activity increased by 55.02%, 65.91%, 73.47%, 82.11%, and 95.73%, respectively, at increasing concentrations of quercetin (from 62.5 to 1000 µg/mL), which was used as a positive control of AGE inhibition ($p \leq 0.01$ for ** and $p \leq 0.0001$ for ****). Considering these results, the arrangement of inhibition activity in three different extracts obtained from *A. caraganae* plant is EtOH/water > EtOH > water. When the biactive components of the extracts obtained from the aerial parts of *A. caraganae* were examined, the order of EtOH/water > EtOH > water was similarly determined in *p*-coumaric acid, isoquercitin, delphindin-3,5-diglucoside, quercitrin and hyperoside and similar phenolic compounds, respectively. This situation is also supported by the information in the literature.[48–51] There are two inhibitors of AGE, which is the main actor in the prognosis of many diseases, one of which is commercially available synthetic inhibitors and the other is phenolic compounds.[50] It has been shown that phenolic compounds, which are abundant and in different derivatives in the natural structure of plants, reduce the levels of reactive oxygen species by scavenging the dicarbonyl compounds of sugars, thus reducing the quantity of AGEs, which target the oxidation of proteins in the cells.[51,52] It has been observed in the literature that plants rich in phenolic compounds such as *A. campylosema*, *A. hirsutus*, *Ribes cucullatum*, and *Phaseolus vulgaris*, also have high AGE inhibition activity. On the other hand, antioxidant results obtained within the scope of the study show parallelism with AGE inhibition. These findings indicate that *A. caraganae* extracts with high antioxidant activity and rich in phenolic compounds have high AGE inhibition and may be natural AGE inhibitors.

2.5 | Determination of cell viability and nontoxic concentration of *A. caraganae*

WST-1 was applied to determine the effect of EtOH, EtOH/water, and water extracts obtained from *A. caraganae* on the viability of HDF cells and to determine the nontoxic concentration. The

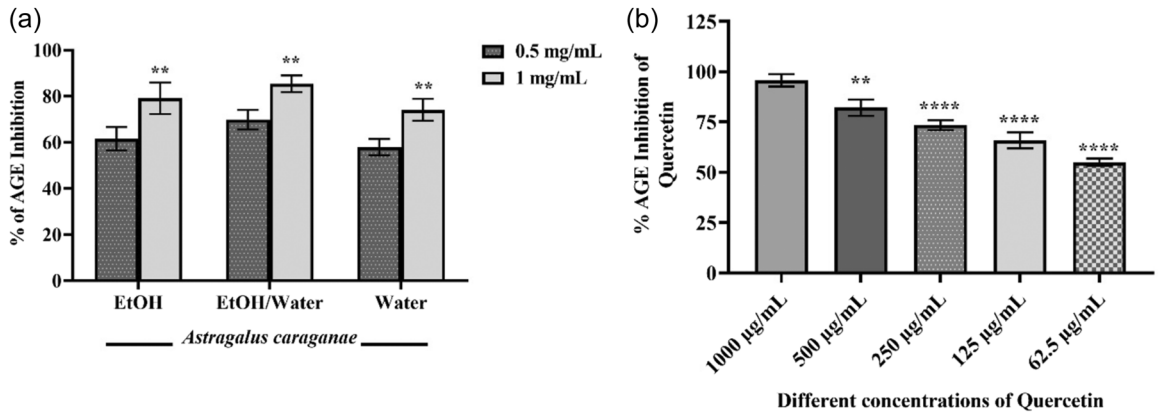

FIGURE 1 Advanced glycation end (AGE) inhibition activity of three extracts from *Astragalus caraganae* at different concentrations and epigallocatechin gallate (EGCG). The inhibitory activity of EtOH, EtOH/water, and water extracts at 0.5 and 1 mg/mL concentrations on AGE was presented in (a), while the inhibitory activity of EGCG at increasing concentration was presented in (b). The statistical analysis was performed with the GraphPad Prism program, **$p \leq 0.01$, ***$p \leq 0.0001$.

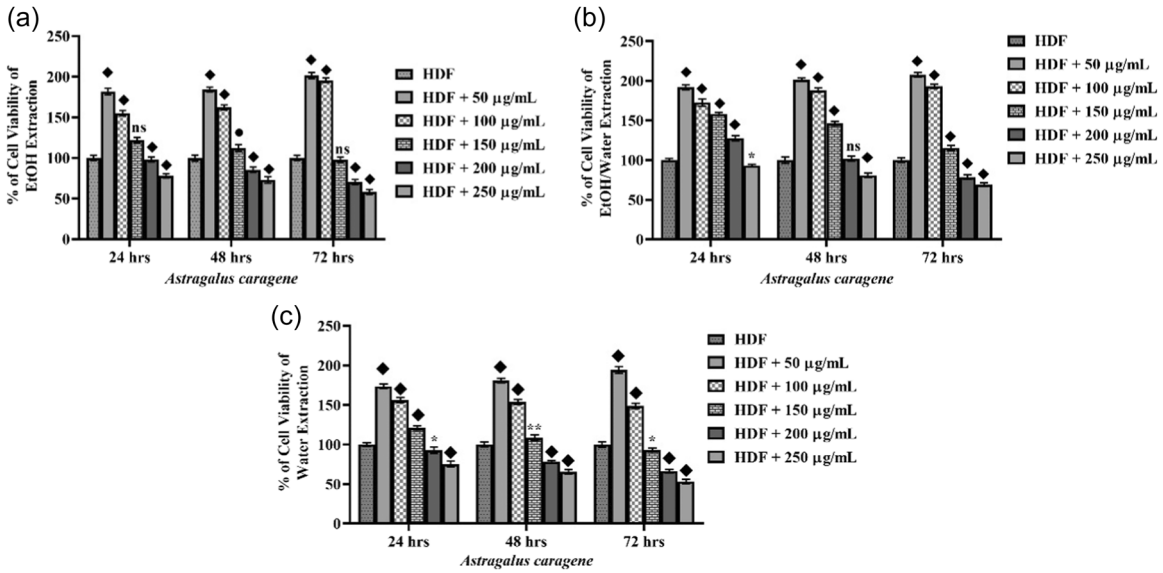

FIGURE 2 Detection of nontoxic concentration of three different extracts of *Astragalus caraganae* on human dermal cell (HDF) cells by WST-1. The nontoxic concentration of the EtOH extract at increasing concentrations at three different time points was presented in (a), the nontoxic concentration of the EtOH/water extract was shown in (b), and the time-dependent nontoxic concentration of the water extract was illustrated in (c). As a result of the statistical analysis, ♦$p \leq 0.0001$, ●$p \leq 0.001$, $^{**}p \leq 0.01$, and $^{*}p \leq 0.05$. Nonsignificant statistical analyses were indicated as ns.

experiments were repeated at least three times independently of each other and their effects on toxicity were checked at 24, 48, and 72 h, respectively, and are presented in Figure 2. All time points were compared among themselves with untreated HDF cells and the viability of this cell group was accepted as 100%. It was observed that the cell viability of EtOH extract obtained from *A. caraganae* at the end of 24, 48, and 72 h, respectively, was 78.25%, 73.01%, and 58.21% at the highest concentration of 250 µg/mL (Figure 2a). Similarly, when untreated HDF cells were compared with HDF cells treated with the highest concentration of EtOH/water extract, cell viability was determined as 191.85%, 172.69%, 158.17%, 127.61%, and 93.23%, respectively, at increasing concentrations at 24 h (Figure 2b). At the end of the 48th hour, cell viability was observed to be 201.28%, 188.35%, 146.23%, 101.92%, and 80.91%, respectively, at increasing concentrations (Figure 2b). At the end of 72 h, cell viability was found to be 207.61%, 193.02%, 115.28%, 78.55%, and 69.14%, respectively, compared to the control untreated-HDF cells (Figure 2b). When similar comparisons were performed for the water extract in Figure 2c, 75.38% viability was detected at the highest concentration at the end of the 24th hour, while this rate was 65.45% for 48 h and 53.17% for 72 h (Figure 2c). While EtOH/water extract's cell viability was 93.23%, even at the highest concentration, the lowest cell survival was found in water extract with 53.17%. In this context, the highest cell viability was listed as EtOH/water > EtOH > water when all time points were compared with the same time point within itself. Within the scope of this study, a working concentration of 200 µg/mL was determined where cell death did not fall below 60% for all determined time points. As indicated in the accumulated data, it has been found that plants regulate the cell cycle thanks to their phenolic compounds.[43,53,54] An earlier study showed that the lost cell cycle and low cell viability level via the lipopolysaccharide-induced ROS mechanism in A549, HUVEC, CRL-1730, and ECV304 cell lines were regained by the administration of *Astragalus membranaceus* to cell lines.[53] These findings can be explained by the high amount of phenolic compounds, bioactive components, saponins, and alkaloids, which *Astragalus* species possess.[4,53–55] Extracts rich in phenolic compounds regulate apoptosis regulation via the p53/p21 pathway and reduce the expression of antiapoptotic Bcl-2 expression, keeping cell proliferation and viability under control. In addition, there is information indicating that phenolic-rich extracts can be associated with telomerase and survivin expression, thus maintaining cell viability.[54,56] In light of this information, the reason why cell viability continues without falling below 50% value in increasing concentration is the high bioactive compounds it has in *A. caraganae*. In addition, decreased cell proliferation depending on concentration and time indicates that there may be a cytostatic effect rather than an absolute cytotoxic effect.[57,58]

2.6 | Effect of *A. caraganae* on genotoxicty

Comet assay was applied to determine the possible genotoxic effect of three different extracts from *A. caraganae* on HDF cells (Figure 3). To compare the negative effect on cells, HDF cells were treated with 500 µM concentration of H_2O_2 for 48 h.[59] Untreated HDF cells were called the control group, while the positive control group was HDF cells treated with epigallocatechin gallate (EGCG) at the

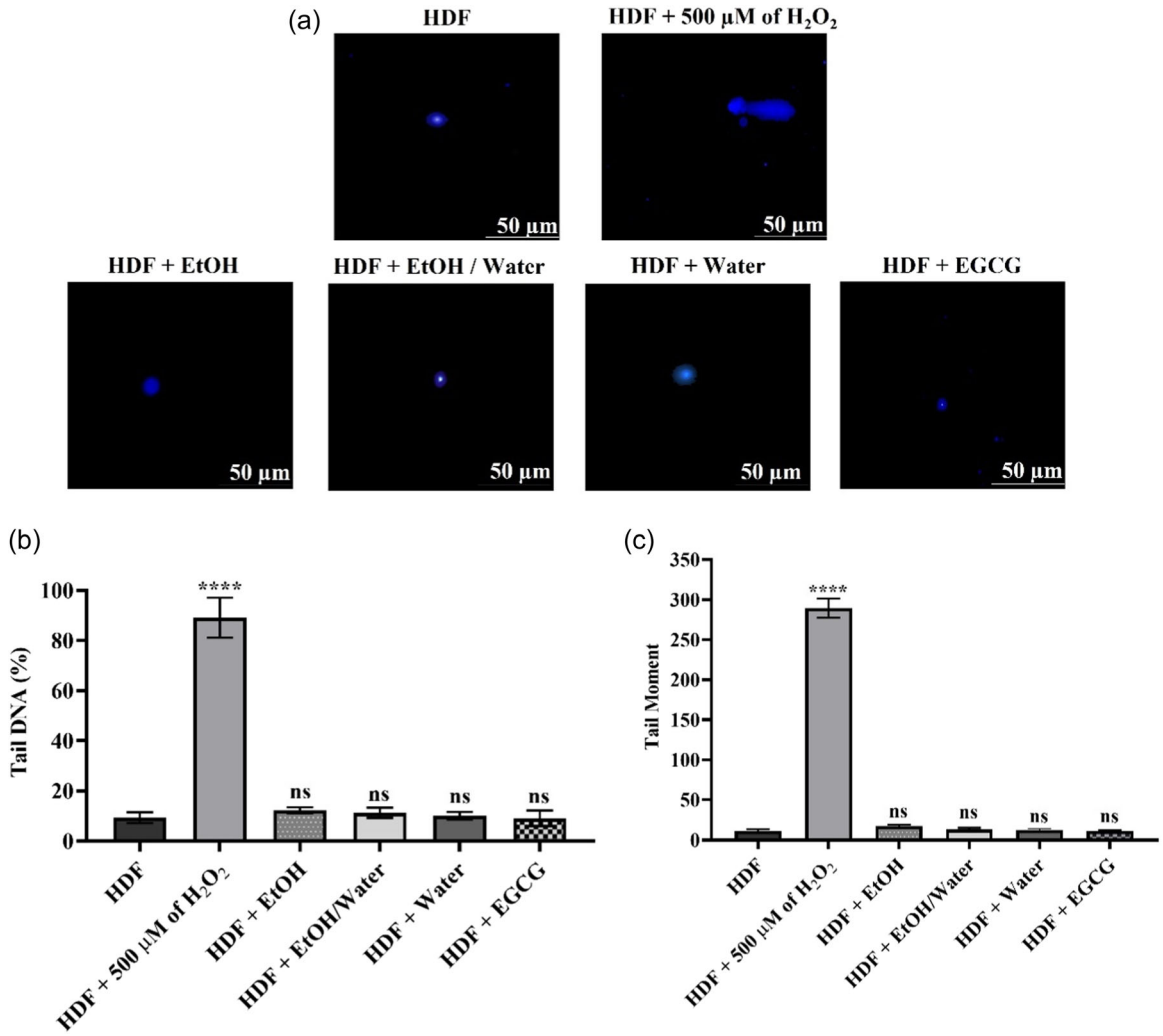

FIGURE 3 Determination of the genotoxic effect of three different extracts from *Astragalus caraganae* on human dermal cell (HDF) cells. Images of untreated-HDF, HDF cells treated with 500 µm H_2O_2, and HDF cells treated with three different extracts under a fluorescence microscope were shown in (a). Epigallocatechin gallate (EGCG) at a concentration of 250 µg/mL was used as a positive control. While the % Tail DNA value was shown in (b), the tail moment was in (c). As a result of the statistical analysis, ****$p \leq 0.0001$, ns, nonsignificance results.

concentration of 250 µg/mL. Sample groups were HDF cells treated with 200 µg/mL concentrations of EtOH, EtOH/water, and water extracts (in Figure 3a). As a result of the statistical analyses, the Tail DNA percentage was presented in Figure 3b, while the Tail Moment value was given in Figure 3c. Compared to untreated HDF and H_2O_2-treated HDF cells, H_2O_2 damaged the cells' DNA, resulting in approximately 9.6 times more Tail DNA formation. Similar comparisons were made for other cells and no statistical difference was found between EtOH-, EtOH/water-, water-treated HDF, and EGCG-treated HDF cells, respectively (in Figure 3b, $p > 0.05$ used for ns). Similar results were obtained in similar comparisons performed in Figure 3c, and no genotoxic effect was observed in three different extracts and EGCG, despite the genotoxic effect created by H_2O_2 (in Figure 3). It indicates that phenolic compounds such as rutin, hyperoside, and isoquercitrin are contained in *A. caraganae*, and play an active role in the protection of cell-DNA integrity.[43] On the other hand, in both phytochemical content and antioxidant results, *A. caraganae* has been found to remove ROS as effectively as the reference substance EGCG and play a crucial role in regulating DNA integrity. In another study with similar results, when ROS donor formaldehyde was applied to bone marrow stem cells, oxidative stress increased and DNA strand breakage occurred. Then, when these formaldehyde-treated stem cells were treated with *Astragalus* polysaccharide, it was observed that the DNA repair mechanism was supported, and the ROS level decreased.[60] The major active compounds in *Astragalus* species play a role in upregulating the nucleotide excision repair (NER) pathway to regain exogenous and endogenous-induced decreased genomic stability and eliminate genotoxicity in cells.[60,61] Bioactive compounds of the *Astragalus* species that trigger the activation of this pathway primarily regulate the synthesis of Excision repair cross completion group 1 (ERCC1) protein, which has an essential role in cellular DNA damage repair, and the endonuclease XPG/-XPF proteins, which this protein forms a complex in the downstream.[60,62] Thus, the ERCC1-XPF complex

activates the NER pathway, which is responsible for cutting the DNA-damaged oligonucleotides, it is cut at the 5–6 nucleotide lower (3′) end of the DNA helix and 20–22 nucleotides higher (5′) end, supporting genetic stabilization.[62] The results presented in the current study are in correlation with the published literature about *Astragalus* species in terms of genotoxicity and DNA instability.

2.7 | Effect of *A. caraganae* on protein synthesis

Within the scope of this study, Western blot technique was used to determine the protein pathways thought to play a role in the H_2O_2-induced ROS mechanism in HDF cells and to determine the changed protein synthesis after the application of three different *A. caraganae* extracts (Figure 4). In this context, phospho-NF-κB, RAGE, BCL-2, BAX, and p-53 protein biosynthesis levels were determined, respectively (Figure 4a). When comparing band intensities, the band thickness of untreated HDF cells was calculated as 1 fold. As a result of the quantitative evaluation based on band density, it was observed that phospho-NF-κB synthesis increased 2.7 times in H_2O_2-treated HDF cells compared to untreated-HDF. When H_2O_2-treated HDF cells were compared with EtOH, EtOH/water, water applied H_2O_2-treated HDF cells, there were 2.13-, 2.39-, and 1.81-fold reduction, respectively (Figure 4b, ♦ used for $p \leq 0.0001$). It was found that phospho-NF-κB synthesis was reduced by 2.09-fold in H_2O_2-treated HDF cells treated with EGCG used as a positive control (Figure 4b, ♦ used for $p \leq 0.0001$). In the RAGE protein, where a similar comparison was made, 2.27 times more RAGE was synthesized after H_2O_2 administration, while RAGE levels decreased 0.95, 1.85, and 0.99 times in H_2O_2 treated-HDFs exposed to EtOH, EtOH/water, water extracts, respectively (Figure 4b, ♦ used for $p \leq 0.0001$). The RAGE value decreased 1.65-fold in EGCG-treated cells used as a positive control. Within the BCL-2 protein level, 2.30 times more protein was expressed in H_2O_2 treated-HDF cells when compared to untreated-HDF cells. When the H_2O_2 treated-HDF cells were compared with the H_2O_2 treated HDFs exposed to EtOH, EtOH/water, and water extracts, it was determined that the BCL-2 protein level decreased by 1.74, 1.87, 1.44 times, respectively (Figure 4b, ♦ used for $p \leq 0.0001$). This decrease was 1.67-fold in EGCG-applied H_2O_2 treaded-HDF cells. At the Bax protein level, where similar comparisons were made, the protein was synthesized 2.92 times less after H_2O_2 application, while it was found that the Bax protein was synthesized 2.85 times more in EtOH treated cells, 3.15 times more in EtOH/water and 1.95 times more for water. The Bax level increased 2.53-fold with subsequent administration of EGCG to H_2O_2-treated HDF cells. The analysis made for p-53 showed that the protein level decreased by 2.31 times after H_2O_2 application, while the P-53 value increased by 2.93 times for EtOH, 3.32 for EtOH/water, and 2.27 times for water, respectively. It was found that p-53 protein biosynthesis increased 2.09 times after EGCG administration. In light of these findings, the molecular mechanism of ROS induced by H_2O_2 was regulated by three different *A. caraganae* (EtOH/water > EtOH > water) applications. Our protein biosynthesis results, which are compatible with the antioxidant experiments in which similar results were observed, gave rise to the idea that our model plant may have a crucial role in protein synthesis with the help of high and various bioactive components. H_2O_2 induced-oxidative stress activates the nuclear factor-κB (NF-κB) signaling pathway and stimulates the downstream/upstream proteins involved in this signaling mechanism.[63] Under normal conditions, NF-κB interacts with inhibitory κB (IκB) proteins in a heterodimer structure and is inactive in the cytoplasm. With the increase of ROS/antioxidant ratio, the reception of inflammatory signals such as lipopolysaccharide or tumor necrosis factor-alpha, NF-κB is phosphorylated and translocated to the nucleus and plays a role in the prognosis of many diseases along with the inflammation process.[64,65] Another protein required for downstream signaling and activation of NF-κB during the oxidative stress period is the AGE receptor protein RAGE. The AGE/RAGE interaction induced by increased ROS level activates NF-κB, an effector of the oxidative stress pathway in cells.[64,66,67] On the other hand, it has been shown in previous studies that the apoptotic mechanism is disrupted in cells with ROS.[68] In the mechanism of apoptosis, called programmed cell death, an increase in the antiapoptotic BCL-2 due to an increase in the amount of ROS, in turn, there is a decrease in the levels of the proapoptotic proteins BAX and p-53, as a result of which the cells become resistant to apoptosis, and the cell cycle is disrupted.[64,68,69] Various plants with high antioxidant potential are used for the improvement and rearrangement of ROS-induced impaired molecular signaling pathways.[5,64,70] In studies with *Astragalus* species, ROS-induced loss of cell cycle in cells is regulated thanks to bioactive components such as various flavonoids, saponins, and alkaloids contained in the plant.[5,71] This information in the literature shows parallelism with the results of Western blot (in Figure 4). It has been observed that the cellular cycle lost with H_2O_2 application is regulated and the apoptosis mechanism is gained, thanks to bioactive components such as gallic acid, rutin, chlorogenic acid, hyperoside, isoquercitin, which are obtained from three different extracts (ethanol, ethanol/water, and water) obtained from the aerial parts of the *A. caraganae*. In addition, it was determined that the RAGE protein level decreased to basal levels thanks to the AGE inhibition activity of *A. caraganae* and decreased NF-κB activation.

2.8 | Determination of intracellular and mitochondrial reactive oxygen levels by DCFDA

The DCFDA experiment was performed to detect the increased cellular ROS level after H_2O_2 treatment and to clarify the roles of three different *A. caraganae* extracts applied subsequently in reducing the ROS level (Figure 5). In the time-dependent experiment, it was determined that the cellular reactive oxygen level increased 12.24 times when H_2O_2 applied for 24 h was compared with untreared-HDF cells ($p \leq 0.0001$ value was symbolized by ****). On the other hand, it was observed that intracellular ROS level decreased by 5.2 after EtOH administration to H_2O_2-treated HDF cells (*** was

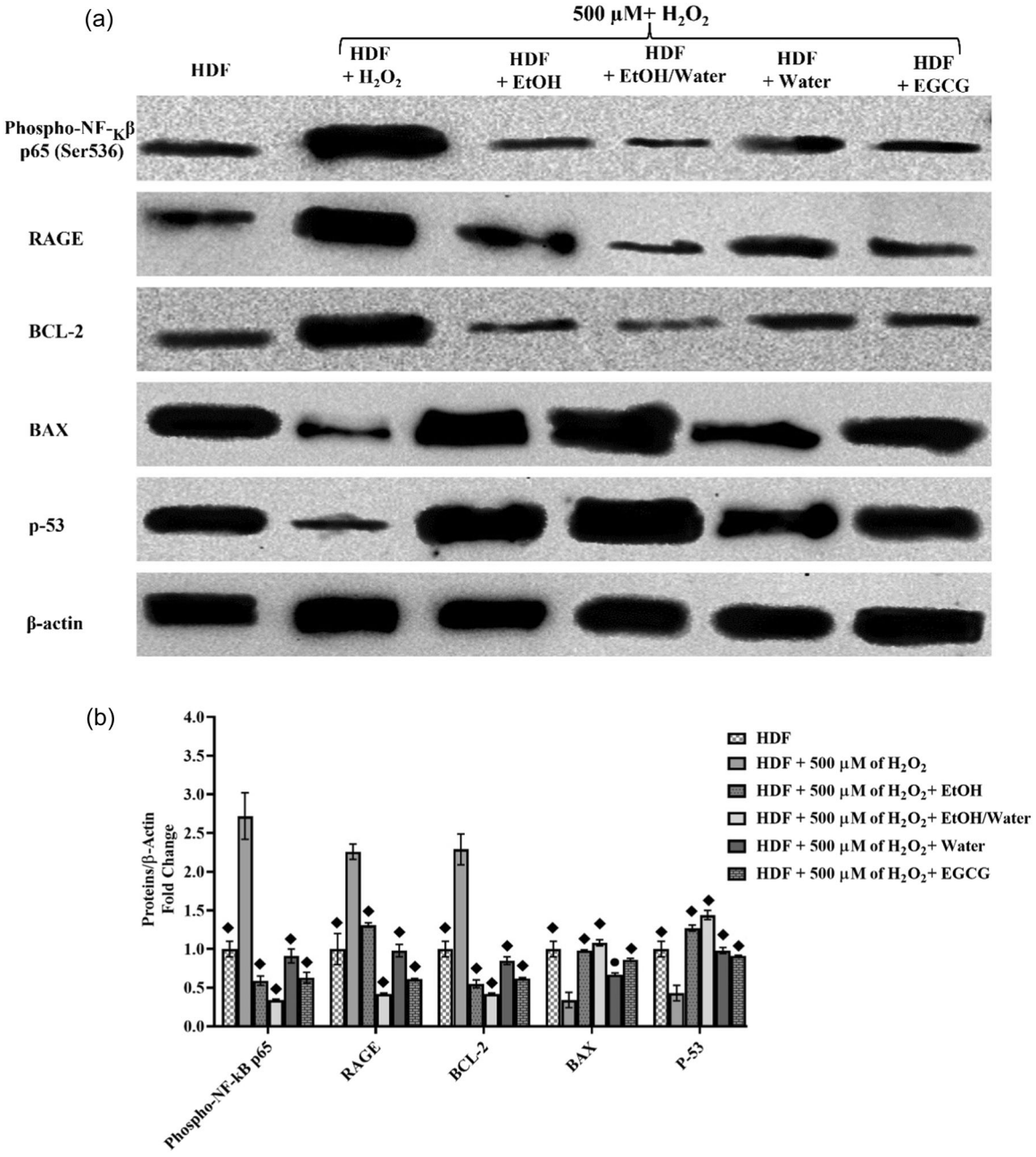

FIGURE 4 Determination of the levels of proteins in the oxidative stress pathway induced by H_2O_2 with Western blot. The effects of EtOH, EtOH/water, and water extracts applied to H_2O_2-treated HDF for 48 h and H_20_2 treated/untreated HDF on phospho- NF-κB, RAGE, BCL-2, BAX, and p-53 protein levels were visualized with the help of ChemicDOc and presented in (a). Beta-actin was used as a loading control. Quantitative analysis of band density was in (b). As a result of statistical analysis, a significant $p \leq 0.0001$ value was demonstrated with ♦ and ● was used for $p \leq 0.001$.

used for $p \leq 0.001$). While ROS reduction value is 7.2 for EtOH/water, it is 4.08 for water. After 24 h administration of positive control EGCG, the level of ROS was calculated to decrease by 7.2 compared to H_2O_2-treated HDF cells ($p \leq 0.0001$ for **** in Figure 5). In comparisons performed at the end of 48 h, the ROS level in cells increased 17.11 times depending on time (**** for $p \leq 0.0001$) with H_2O_2 application. It was determined that the intracellular ROS level decreased by 11.35 after EtOH extract obtained from *A. caraganae*, 14.38 after EtOH/water application and by 11 for water. It was found that this value decreased by 15 after EGCG administration ($p \leq 0.0001$ for **** in Figure 5). As known, mitochondria regulate the dynamics and balance of ROS in cells.[72,73] An imbalance in intracellular ROS levels may occur due to increased reactive oxygen species in mitochondria.[74] This condition may cause cells to undergo oxidative stress and weakening cells against many different diseases.[74,75] Even sometimes in cells that cannot overcome oxidative stress, apoptosis may be disrupted and the cells may enter the process of becoming cancerous.[74] For this reason, it is very

important to use antioxidant-rich products to restore the ROS balance of cells and establish cell homeostasis.[76–79] *A. caraganae* was found to have high bioactive compounds in phytochemical content analysis and antioxidant experiments. In particular, the extracts obtained from *A. caraganae* contain bioactive components such as gallic acid, rutin, chlorogenic acid, hyperoside, isoquercitin (EtOH/water > EtOH > water), which reduces H_2O_2-induced oxidative stress in cells.

2.9 | Effect of *A. caraganae* on MMP-2/MMP-9 gene expressions and activations

The effects of the gelatinase groups MMP-2 and MMP-9, which changed after the application of three different extracts from *A. caraganae* to H_2O_2 treated-HDF cells, on gene expressions are shown in Figure 6 and enzyme activity are presented in Figure 7. For quantitative analysis of the expressions of MMP-2 and MMP-9, 18s rRNA, a housekeeping gene, was used.[64,70,80] As a result of the analysis, the MMP-2 level increased 1.98 times when untreated HDF cells were compared with H_2O_2 treated-HDF cells (in Figure 6a). It was determined that MMP-2 gene expressions decreased by 1.70, 1.95, and 1.61 times, respectively, after EtOH, EtOH/water, and water extracts applied to HDF cells with H_2O_2. MMP-2 gene level decreased 1.58-fold following administration of positive control EGCG (**** for $p \leq 0.0001$ in Figure 6a). On the other hand, in the comparison made for MMP-9 gene expression, it was found that the level increased 3.50 times after H_2O_2 administration. The change in MMP-9 gene expression was determined as 2.64 fold decrease in EtOH, 3.4-fold in EtOH/water, and 2.11-fold decrease in water

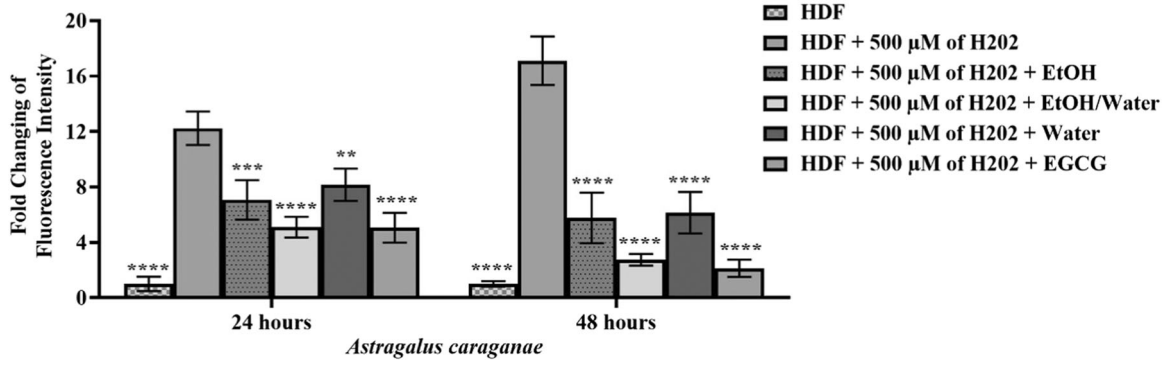

FIGURE 5 Detection of H_2O_2-induced cellular oxidative level by Dichlorodihydrofluorescein diacetate (DCFDA). After applying three different extracts from *Astragalus caraganae* to human dermal cell (HDF) cells treated with or without H_2O_2, the changing intracellular and mitochondrial reactive oxygen level was displayed in this figure. Epigallocatechin gallate (EGCG) was used as a positive control. In the statistical analysis performed with the GraphPad Prism program, the value of $p \leq 0.0001$ was symbolized by ****.

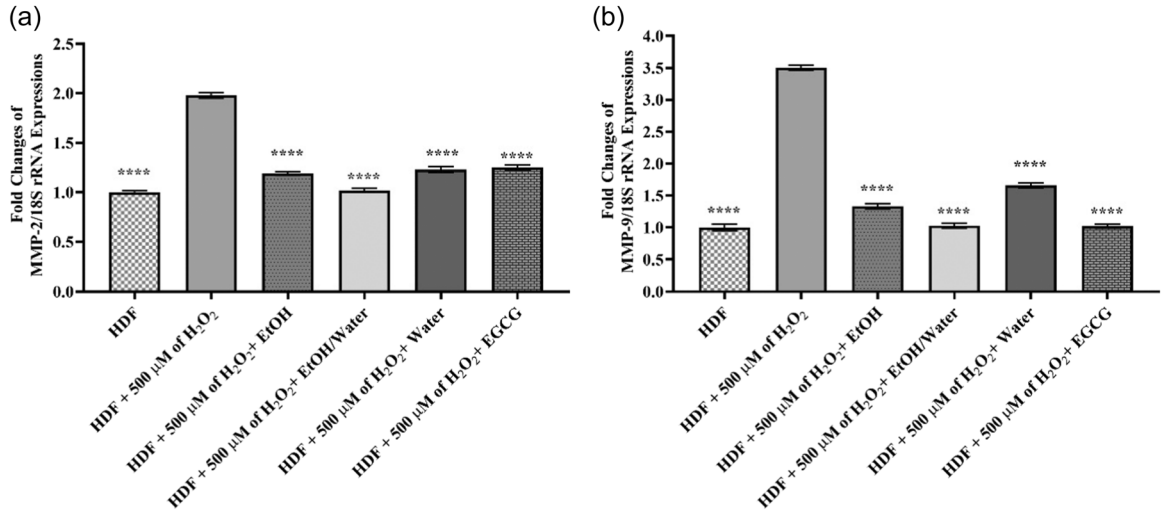

FIGURE 6 Determination of altered MMP-2 and MMP-9 gene expressions in human dermal cell (HDF) cells treated with three different extracts from H_2O_2 and *Astragalus caraganae*. The effect of *A. caraganae* applied after triggering oxidative stress in HDF cells on MMP-2 gene expression was presented in (a), while MMP-9 gene expression was shown in (b). Epigallocatechin gallate (EGCG) was used as a positive control. For the statistically significant value, **** was used $p \leq 0.0001$.

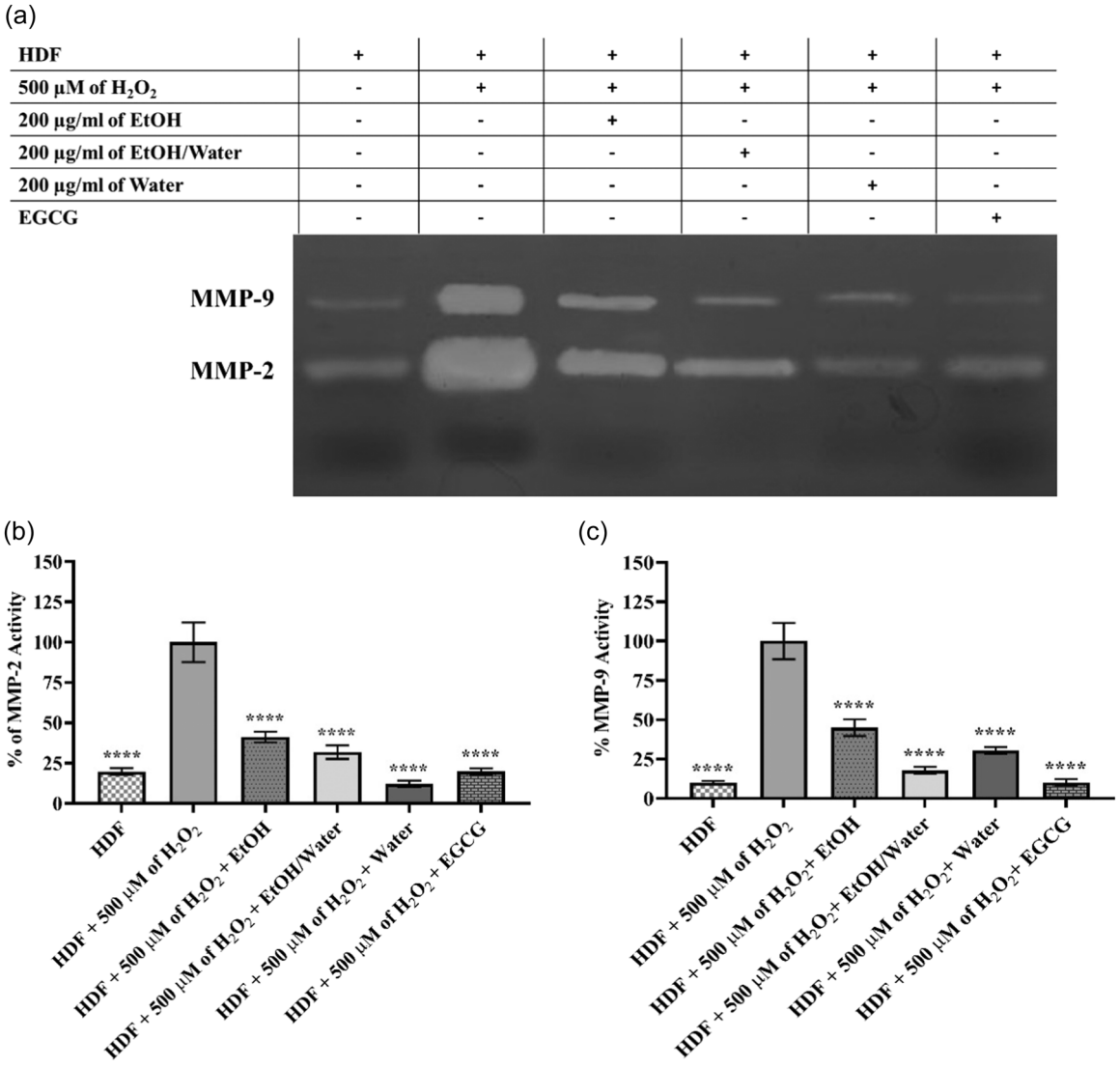

FIGURE 7 Altered MMP-2 and MMP-9 biosynthesis after treatment of *Astragalus caraganae* extracts to H_2O_2-treated-/untreared-human dermal cell (HDF) cells (a). The effect of *A. caraganae* applied after triggering oxidative stress by H_2O_2 in HDF cells on MMP-2 activity was shown in (b), while its effect on MMP-9 activity was illustrated in (c). For the statistically significant $p \leq 0.0001$ value was indicated with ****. Epigallocatechin gallate (EGCG) was used as a positive control.

(**** for $p \leq 0.0001$ in Figure 6b). There was a 3.43-fold reduction in MMP-9 gene expression after administration of EGCG to H_2O_2-treated cells. In the zymography experiment, in which similar comparisons were made, in which enzyme activity of MMP-2 and MMP-9 was determined, the application of H_2O_2 increased the activity in cells by 80.17% and 90.01%, respectively (Figure 7a,b). When EtOH, EtOH/water, and water extracts were applied to HDF cells treated with H_2O_2, it was observed that MMP-2 levels decreased by 58.83%, 68.21%, and 87.83%, respectively. This value was found to be 80% reduction after EGCG administration (in Figure 7a). MMP-9 activity decreased by 54.98%, 82.10%, and 69.54%, respectively, when EtOH, EtOH/water, and water extracts were applied to H_2O_2 treated-HDF, while it decreased by 90% after EGCG administration (in Figure 7b, **** for $p \leq 0.0001$). As it is known, MMPs that play a role in the regulation of the extracellular matrix can be overexpressed due to oxidative stress.[81] Overexpressed MMPs degrade proteins in the ECM, causing damage to connective tissue, the development of many diseases, and an increase in the invasion/migration potential of cancerous cells.[82] In studies conducted with different *Astragalus* species, it has been shown that ECM integrity lost in photoaging caused by oxidative stress, especially in Chinese medicine, is regulated by the high phytochemical content of *A. membranaceus*.[83] In the study, which showed that gastric cancer invasion and migration increased due to impaired ECM integrity with the help of highly synthesized MMPs, it was determined that after *A. membranaceus* treatment, cells synthesized MMP at basal level and with the help of this, migration, invasion and survival potentials of cancerous cells were inhibited.[83] *A. complanatus* was applied to remodel ECM integrity and improve fibrotic tissue formation. Another study observed that the MMP

levels of the cells were decreased and apoptosis was regulated, thanks to the use of an isoflavone species obtained from the *Astragalus* species in ovarian cancer.[84] In another study presented in the literature, liver damage was created by oxidative stress by applying malondialdehyde (MDA) and superoxide dismutase to liver cells. As a result, an increase in MMP levels was observed, and the cells formed fibrotic tissue. *A. complanatus* was applied to these damaged cells to remodel ECM integrity and remove fibrotic tissue formation. Thanks to the high flavonoid content of the plant, it was determined that MMP levels decreased and ECM modulation was regained.[85] In the same study, it was observed that tissue inhibitors of metalloproteinas, which are responsible for the regulation and inhibition of MMPs, inhibited increased MMP synthesis due to oxidative stress, and TIMP-1 protein level increased after *A. complanatus* treatment.[85] The data obtained in parallel with this information indicate that ECM integrity, which was impaired due to oxidative stress induced after H_2O_2 administration, was restored with the application of *A. caraganae*. Both RT-PCR results and zymography enzyme-activated experiments performed for MMP-2 and MMP-9 have revealed that all three extracts obtained from *A. caraganae* contribute to ECM remodeling under in vitro conditions.

2.10 | Molecular docking

All docked compounds were found to bind to all five enzymes, with an apparent preference for AChE and BChE for most compounds (Figure 8). The detailed protein–ligand interactions show how some selected compounds engaged the active site amino acid residues in various interactions. Delphinidin-3,5-diglucoside was accommodated in the active site of AChE via mainly, multiple H-bonds and van der Waals interactions all over the catalytic channel. Also, a couple of hydrophobic interactions in the middle of the tunnel reinforced the binding (Figure 9a). Similarly, rutin spanned the catalytic cavity of BChE, mainly H-bonds, van der Waals interactions, and a couple of π–π stacked interactions (Figure 9b). Interestingly, relatively smaller compounds, isoquercitrin and hyperoside fit in the relatively narrow active sites of tyrosinase (Figure 9c) and alpha-amylase (Figure 9d), respectively, via multiple H-bonds and π–π stacked interactions, a hydrophobic interaction, and several van der Waals interactions. Also, chlorogenic acid is completely buried in the catalytic channel of glucosidase, forming key interactions comprising multiple H-bonds, a hydrophobic interaction, as well as a couple of van der Waals interactions, thereby contributing to the overall binding strength (Figure 9e).

Finally, to predict the anticancer properties, each compound was docked into MMP-9—a protein that plays a crucial role in cancer invasion, angiogenesis, and metastasis[86]; and into BCL-2—a protein that plays vital roles in cell death via regulating apoptosis.[87] Delphinidin-3,5-diglucoside bound well to both MMP-9 (Figure 10a) and BCL-2 protein via multiple H-bonds and van der Waals interactions, and a couple of hydrophobic interactions (Figure 10b). Taken together, H-bonds are the key interactions with which the selected bioactive compounds from *A. caragene* extracts bind to, and potentially inhibit these targets.

3 | CONCLUSION

In this study, different extracts of *A. caraganae* were studied for their chemical profile and pharmacological properties. The polarity of the solvents used for the different extracts were found to have an effect on the phytochemical extraction as well as their bioactivities. For instance, the least polar solvents extracts (*n*-hexane and dichloromethane) yielded less TPC and TFC in spectrophotometric assays and less total bioactive contents, as revealed by HPLC-MS/MS. The polar extracts were found to be richer in antioxidant compounds such as rutin and phenolic acids, such as *p*-coumaric and chlorogenic acids. Moreover, similar trends were obtained in the antioxidant assays, especially in terms of their radical scavenging and reducing power. The antienzymatic effect of the extracts, however, varied for the different enzymes. While all extracts were found to be dual inhibitors of the diabetic

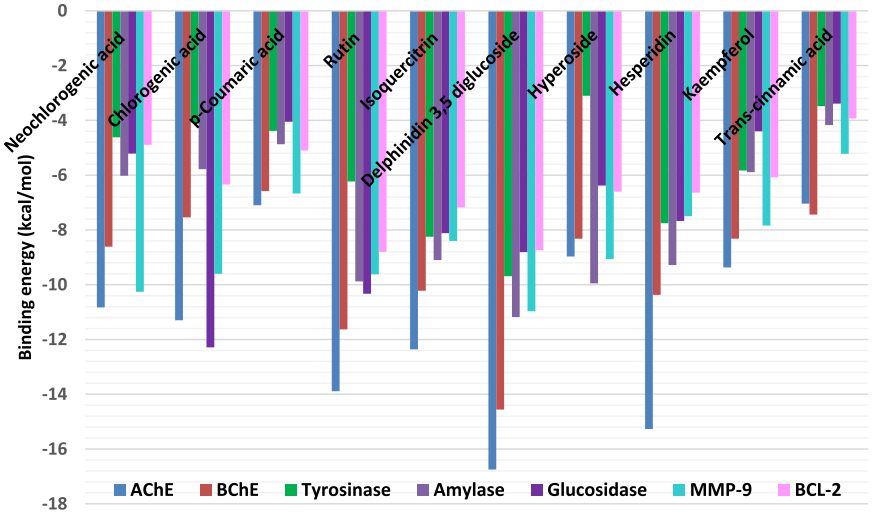

FIGURE 8 Binding energy (docking) scores of the phytochemical compound from *Astragalus caragene* extracts.

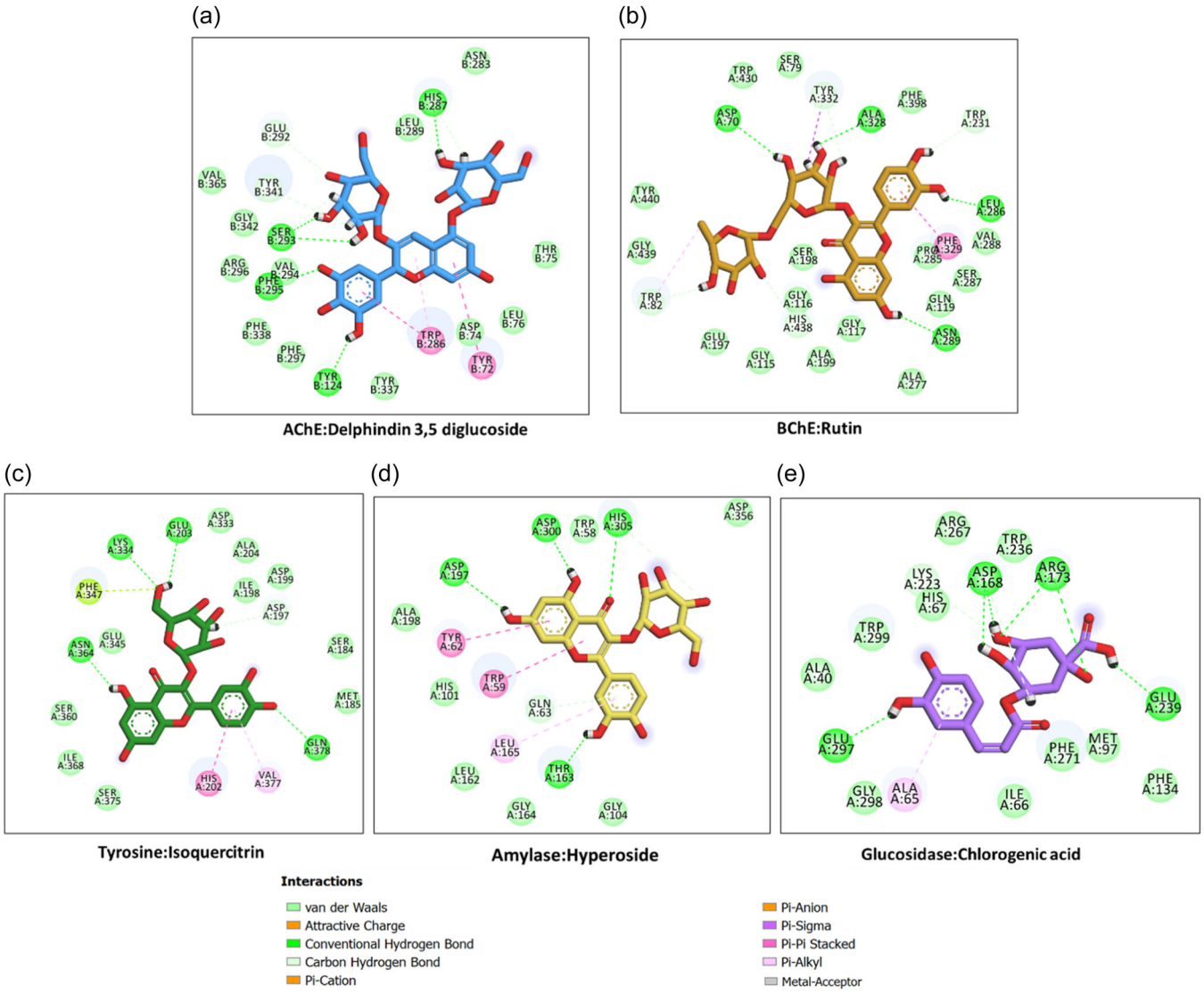

FIGURE 9 Protein–ligand interaction: (a) acetylcholinesterase (AChE) and delphinidin-3,5-diglucoside (b) butyrylcholinesterase (BChE) and rutin, (c) tyrosinase and isoquercitrin, (d) amylase and hyperoside, and (e) glucosidase and chlorogenic acid. These phytochemical compounds were extracted from *Astragalus caraganae*.

enzymes, a few of them did not show inhibition against the other enzymes. As a result of in vitro experiments, it was found that three extracts obtained from *A. caraganae* have AGE inhibition, which plays an active role in both sugar metabolism and oxidative stress pathway, thanks to their high and various phytochemical contents. While it was observed that *A. caraganae* applied to HDF cells at different concentrations had neither genotoxic nor cytotoxic effects on HDF cells, it was found that they could create a cytostatic effect depending on the concentration. It has been observed that phospho-NF-κB, RAGE, BCL-2, BAX, and p-53 proteins in the ROS-induced oxidative stress pathway, respectively, return to healthy cell profiles following administration of *A. caraganae*. This indicates that cellular molecular pathways can be regulated, and oxidative stress can be eliminated thanks to *A. caraganae*. In addition, it has been revealed that *A. caraganae* can remodeling the extracellular matrix by acting as an MMP inhibitor in the rearrangement of the deteriorated ECM structure. To the best of our knowledge, this is the first report to provide an overview of the chemical profile, bioactive contents and in vitro pharmacological effects of a variety of solvent extracts derived from this species belonging to the well-recognized *Astragalus* genus. Hence, this study could help to contribute to the better understanding of this medicinal plant in traditional medicine and its potential in modern medicine.

4 | EXPERIMENTAL

4.1 | Plant materials

In the summer of 2021, we collected aerial parts of *A. caraganae* (Harput, Mardin, 1400 m) in Turkey. The plant specimens were

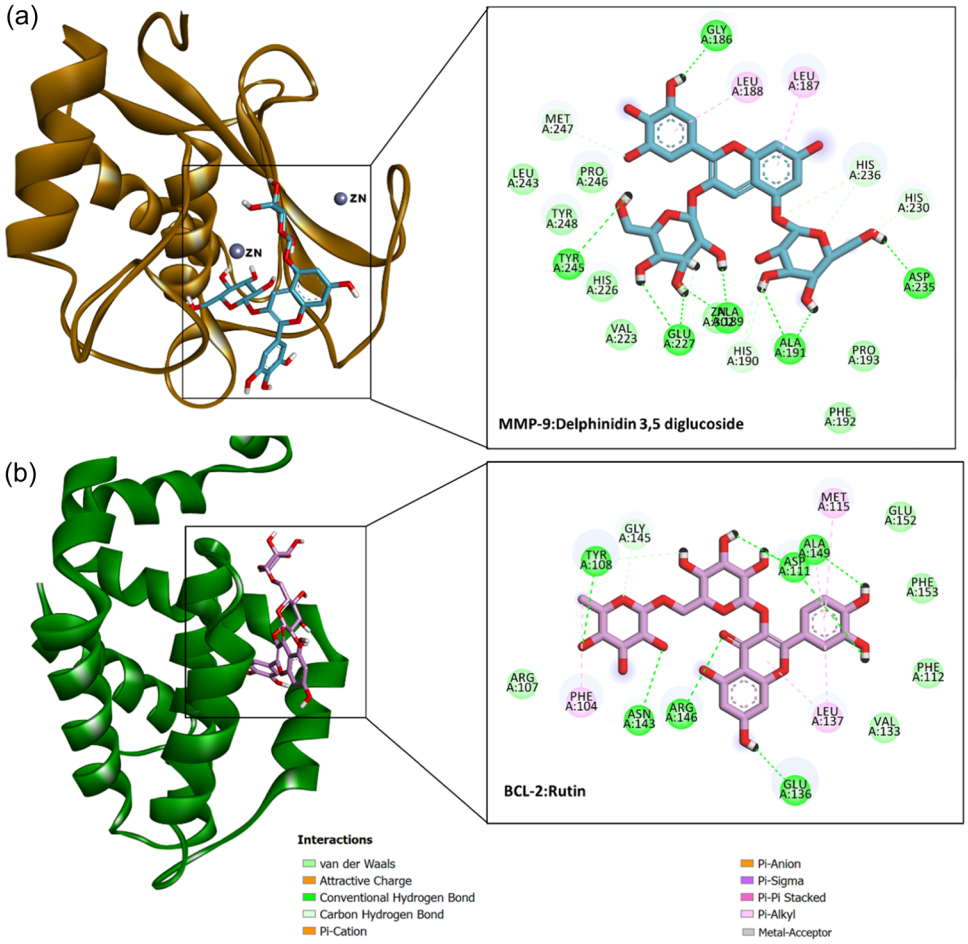

FIGURE 10 Protein–ligand interactions: (a) MMP-9 and delphinidin-3,5-diglucoside (b) BCL-2 and rutin. These phytochemical compounds were extracted from *Astragalus caraganae*.

identified by one of our coauthors, Dr. Ugur Cakilcioglu, and one specimen from the plants was deposited at the Munzur University herbarium. Before extraction, the plant materials were carefully washed with tap and distilled water to eliminate any soil and contaminants. After being air-dried for 10 days (in shade at room temperature), the aerial parts were powdered.

4.2 | Extraction of samples

To prepare extracts, we employed different solvents: *n*-hexane, ethyl acetate, dichloromethane, ethanol, ethanol/water (70%), and water. The maceration method was used for the organic extracts, whereby 10 g of plant material was mixed with 200 ml of each solvent and left for 24 h at room temperature. Using Whatman 1 filter paper, the mixtures were then filtered, and the solvents were eliminated using a rotary-evaporator. The water extracts, on the other hand, were prepared by infusing 10 g of plant material in 200 mL of boiled water for 15 min, followed by filtration and lyophilization for 48 h. All extracts were kept at 4°C until analysis.

4.3 | Total quantification of phenolics and flavonoids

We determined the total phenolic and flavonoid content of the extracts using the Folin-Ciocalteu and $AlCl_3$ assays, respectively, according to Zengin and Aktumsek's protocol. The results of these tests were reported in terms of gallic acid equivalents (mg GAE/g dry extract) and rutin equivalents (mg RE/g dry extract).[88]

4.4 | HPLC-MS analysis

We analyzed the contents of 37 phenolic compounds from different plant extracts, which belong to the chemical classes of phenolic acids, flavonols, flavan-3-ols, flavones, proanthocyanidins, anthocyanins, and nonphenolic acids. The analysis was conducted using a modified version of the method proposed by Mustafa et al.[89] To prepare the samples for HPLC-MS/MS analysis, the dried extracts were dissolved in methanol at a concentration of 5 mg/mL and sonicated for 2 min at room temperature. The solutions were then filtered using a 0.2 µm

syringeless filter before injection into the HPLC-MS/MS system. HPLC analysis was performed using the Agilent 1290 Infinity series, which was coupled with the Agilent Technology Triple Quadrupole 6420 in both positive and negative electrospray ionization (ESI) modes. All analytical details are given in supplemental materials.

4.5 | Assays for antioxidant and enzyme inhibition

We analyzed the extracts for a range of antioxidant and enzyme inhibitory activities, including, CUPRAC, DPPH, and ABTS radical scavenging, metal chelating activity (MCA), FRAP, phosphomolybdenum (PBD), and inhibition of amylase, tyrosinase, glucosidase, AChE, and BChE. We employed the previously described methods to evaluate these activities.[90] Each sample was analyzed three times. We presented all data as mean ± standard deviation and subjected them to statistical analysis using analysis of variance (ANOVA). For significant differences in the data ($p < 0.05$), we conducted post-hoc tests using the Tukey method. All statistical analyses were performed using Graph Pad 9.0.

4.6 | AGE products

AGE products are a nonenzymatic reaction that occurs as a result of the glycosylation of macro and micro molecules in the body, especially proteins and lipids, by entering into an irreversible reaction with sugars.[47,91,92] AGE inhibition activity was determined at 0.5 and 1 mg/mL concentrations of ethanol, ethanol/water and water extracts obtained from the aerial parts of *A. caraganae*. In this context, the extracts at two different concentrations were mixed with 10 mg/mL bovine serum albumin (BSA) and 1 mL 0.5 M glucose; following this step, they were incubated at 55°C for 40 min. Then, fluorescence measurement was performed with Thermo Scientific™ Varioskan™ LUX at the wavelength of 370/440 nm. Different concentrations of the quercetin standard were used as reference substances in the experimental setup. The experiment was repeated three times independently on black 96-well plates not to obtain a background.[43,70]

4.7 | Cell culture and WST-1

Human derma fibroblast cells (HFD), commercially purchased from ATTC, were selected as the model cell line of the in vitro study. The cells were cultured in the complete Dulbecco's modified Eagle medium (DMEM) obtained by adding 10% fetal bovine serum and 1% penicillin-streptomycin to the high-sugar DMEM. A humid, sterile mammalian cell incubator containing 5% CO_2 and 95% O_2 at 37°C was used while cells were grown. When the cells covered approximately 85% of their growth plates, some of them were passaged with a detachment solution containing 0.25% Trypsin/0.53 mM EDTA. Then, they were stockpiled with liquid nitrogen for use in further experiments.[93] Within the scope of the study, WST-1 test was applied to determine the noncytotoxic concentrations of ethanol, ethanol/water and water extracts obtained from the aerial parts of the *A. caraganae* plant on HDF cells. In the WST-1 test, which is based on the principle of conversion of tetrazolium salts reduced by mitochondrial enzymes in cells into formazan crystals, cell viability, and cytotoxicity analysis are performed by WST-1.[94] After the cells were seeded and adhered to 96 well plate at 5000/well density, three different *A. caraganae* extracts were applied to the cells at increasing concentrations (from 50 to 250 µg/mL) for 24, 48, and 72 h, respectively. Following the expiration of the incubation period at each time point, cells were treated with 50 µL of WST-1 reagent diluted 1:10 with serum-free medium (SFM) DMEM for 60 min, and then absorbance was measured with Thermo Scientific™ Varioskan™ LUX at 570 nm wavelength.[43,70,93]

4.8 | Comet assay

H_2O_2 at a concentration of 500 µM was treated with cells for 48 h to create a genotoxic effect in HDF cells and to use it as a negative control.[59] On the other hand, while nontreated HDF cells were used as the control group, the cells were treated with ethanol, ethanol/water, and water extracts at a concentration of 200 µg/mL for 48 h to detect the genotoxic effect of *A. caraganae* on HDF cells. In the experimental setup, 250 µg/mL of epigallocatechin gallate (EGCG) was used as a positive control. 200 cells from each cell group were mixed with 1% concentration low melting point agarose and spread on the slide. At the end of the incubation, the slides were incubated with lysis buffer for 1 h at +4°C.[43,93] As a next step, the slides were run on a cold alkaline solution at 25 volts for 20 min. Afterward, the slides were washed with 0.4 M Tris-HCl, neutralized buffer at pH 7.5 and visualized with DAPI. ZEISS Imager A2 Axiocam 305 color fluorescent microscope was used to measure DNA breaks, and CaspLab software program was used for quantitative analysis.[43,93]

4.9 | Target proteins determination with Western blot

HDF cells at a density of 300,000 in a six-well plate treated or not treated with H_2O_2 were incubated with nontoxic concentrations of *A. caraganae* extracts for 48 h. Afterward, commercial RIPA buffer was used for total protein isolation in all HDF cell groups, and protein concentrations were determined by the Lowry method. The varying levels of phospho-NF-κB, RAGE, BCL-2, BAX, and p-53 proteins in these obtained whole-cell lysates were separated with the help of a 13% concentration of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). B-actin was used as equal loading control for proteins.[64,70,80] Separated proteins in the electric field were transferred to a 0.22 µm PVDF (polyvinylidene difluoride) membrane and then treated with their respective antibodies for 16 h, followed by incubation with HRP-labeled secondary antibodies for

2 h at room temperature. After this step, the target proteins were treated with an ECL solution containing horseradish peroxidase (HRP) substrate, and thus they became visible by chemiluminescence reaction. While band images on the membrane were detected with the Bio-RAD ChemiDoc XRS + system, the Gel tab of the ImageJ program was used for quantitative analysis of band intensities.[70,80]

4.10 | Measurement of intracellular and mitochondrial reactive oxygen levels

2',7'-Dichlorodihydrofluorescein diacetate (CM-H2DCFDA) reagent was used to detect changing cellular ROS levels in untreated HDF, H_2O_2 treated HDF, and three different *A. caraganae* extracts applied to H_2O_2 treated HDF respectively, within the scope of the study.[95] HDF cell groups were seeded at a density of 25,000/well in black-bottomed 96 well-plate and were incubated for 24 h in a mammalian cell oven for attachment. Then, complete DMEM was removed with 1× kit solution and 100 µL of DCFDA reagent was added to the cells and incubated at 37°C for 45 min in the dark. Following the end of the incubation, fluorescence measurements were made at Ex 485/Em 535 nm wavelength.[96]

4.11 | Quantitative real-time reverse transcription-PCR

Presented in the in vitro experimental setup, the qRT-PCR method was applied to test the changes in MMP-2 and MMP-9 gene expressions in untreated-HDF, H_2O_2-treated HDF, and three different *A. caraganae* extracts applied to H_2O_2-treated HDF cells, respectively. First of all, after 48 h of incubation of the cell groups in the experimental setup, their mRNAs were isolated with commercially available peqGold Trizol. Then, the Sensiscript cDNA kit was purchased from Qiagen company, and a defined protocol was followed to obtain cDNA. The gene expression for MMP-2 and MMP-9 was determined by combining the primers, which are identical to the obtained cDNA, in the BIO-RAD C1000 Touch Thermal Cycler device at appropriate temperatures.[64,70] A housekeeping gene 18S rRNA was used for relative quantitative analysis.[70,80]

4.12 | Zymography

The gelatin zymography technique was used to define the damaging effect of H_2O_2 at a concentration of 500 µM applied to HDF cells on extracellular matrix integrity and to detect the change in MMP-2/-9 enzyme activities. In the experimental setup, untreated-HDF cells were used as the control group, while HDF cells treated with H_2O_2 were used as a negative control. In addition, to elucidate the role of three different extracts of *A. caraganae* in the regulation of ECM integrity, they were applied to H_2O_2-treated HDF cells for 48 h and were included in the experimental group. In this context, the optimized protocol setup that we brought to the literature before the zymography experiment was performed.[43,64,70,93] ImageJ program was used for quantitative analysis of band intensities in which enzyme activity was detected.

4.13 | Molecular modeling

The crystal structures of target proteins: AChE (PDB ID: 6O52),[97] BChE (PDB ID: 6EQP),[98] and alpha-amylase (PDB ID: 1B2Y),[99] matrix metallopeptidase 9 (MMP-9) (4WZV),[100] and B-cell lymphoma 2 (BCL-2) (6QGH),[101] were retrieved from the protein data bank (PDB) (https://www.rcsb.org/). However, since the crystal structures of human tyrosinase and glucosidase are not available, those of *Priestia megaterium* tyrosinase (6QXD)[102] and *Mus musculus* alpha-glucosidase (7KBJ)[103] were used as templates to construct their human models using UniProt sequences P14679, and P0DUB6, respectively. The detailed procedure of homology modeling has been described previously.[104] All protein structures were prepared according to the protocol described in Ozturk et al.[105] The 3D structures of selected ligands were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and geometry optimization was done using Frog2 webserver (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::Frog2).[106] The cocrystal ligand in each complex was used to define the docking grid box dimension and coordinates using AutoDock Tools 1.5.6, and each ligand was docked into the binding pocket of each protein using AutoDock 4.2.6 (https://autodock.scripts.edu).[107] Details of the docking protocol have been described in refs.[108–110] The docking (binding energy) score of each ligand against each protein was calculated, and protein–ligand interactions were visualized using Biovia Discovery Studio Visualizer v4.5 (https://discover.3ds.com/discovery-studio-visualizer-download)

4.14 | Statistical analysis

The effects of *A. caraganae* on AGE inhibition activity, MMP-2/−9 biosynthesis (zymography assay), MMPs gene expressions (RT-PCR) and genotoxic effects (Comet assay) were analyzed by multiple comparisons in the one-way ANOVA tab of the GraphPad Prism 9.5.1 software program. In addition, two-way ANOVA multiple comparisons in the same software program were used in the analysis of Western blot, WST-1, and DCFDA test results presented in in vitro experiments. Antioxidant and enzyme inhibitory assays were performed in triplicate, with the data expressed as mean ± standard deviation. Statistical analysis (one-way ANOVA with Tukey's post-hoc test) was performed with Xl Stat 2016; $p < 0.05$ was considered significant.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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