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The effect of *Glycyrrhiza glabra* (Licorice root) extracts on inhibition of $3Cl^{pro}$

Glycyrrhiza glabra (meyan kökü) Ekstraklarının $3Cl^{pro}$ üzerine inhibisyon etkisi

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ABSTRACT

Aim: SARS-CoV-2 virus causes COVID-19, a disease characterised by high mortality rates and severe symptoms such as acute respiratory failure. Specific natural compounds with flavonoid structures have been shown to inhibit 3-chymotrypsin-like protease (3-CLpro), which is crucial for the replication of SARS-CoV-2. Flavonoids interact with the active site of the enzyme, leading to inhibition. The aim of this study was to determine the inhibitory concentrations of flavonoid molecules on 3-CLpro and to obtain the most effective licorice (Glycyrrhiza glabra L.) extracts rich in these molecules.

Materials and Methods: For the extraction of active compounds, 5 different methods were used: ethanol soaking, soaking in water, boiling in water, microwave assisted extraction and ultrasonic assisted extraction. The concentrations of active compounds were determined by LC-MS/MS method. Antioxidant, anti-inflammatory and 3 Clpro inhibition capacities of the extracts were determined by colourimetric methods.

Results: Especially ethanol extracts of liquorice root showed the highest TEAC, FRAP and DPPH levels when evaluated in terms of antioxidant parameters. The strongest 3-CLpro enzyme inhibitory effects were observed in liquorice root extracts obtained by soaking at 80 °C for 6 h, ultrasound assisted soaking for 20 min, soaking in water at 40 °C for 24 h, soaking in 60% ethanol and soaking in 80% ethanol. It was determined that liquorice showed an inhibitory effect on 3-CLpro.

Conclusion: In our study, well-studied bioactive compounds, such as glycyrrhizin and glycyrrhetinic acid, as well as the less common phenolic acid and flavonoid content in liquorice were examined. Among the compounds analysed in liquorice, apigenin, pelargonin, cyanidin, maleic acid, ethyl ferulate and chlorogenic acid were the most abundant. Ethanol extracts of liquorice showed higher concentrations of phenolic and flavonoid compounds associated with increased antioxidant and anti-inflammatory activities.

Keywords: Sars-CoV-2, *Glycyrrhiza glabra* (licorice), 3-CLpro, extraction.

ÖZ

Amaç: *SARS-CoV-2 virüsü, yüksek ölüm oranları ve akut solunum yetmezliği gibi ciddi semptomlarla karakterize bir hastalık olan COVID-19'a neden olmaktadır. Flavonoid yapılara sahip spesifik doğal bileşiklerin, Sars-CoV-2'nin replikasyonu için çok önemli olan 3-kimotripsin benzeri proteazı (3-CLpro) inhibe edebildiği gösterilmiştir. Flavonoidler enzimin aktif bölgesi ile etkileşime girerek inhibisyona yol açar. Bu çalışmanın amacı, flavonoid moleküllerinin 3-CLpro üzerindeki inhibitör konsantrasyonlarını belirlemek ve bu moleküller açısından zengin olan meyan kökü (Glycyrrhiza Glabra L.) ekstraktlarının en etkili olanlarını elde etmektir.*

Gereç ve Yöntem: *Aktif bileşiklerin ekstraksiyonu için etanolde bekletme, suda bekletme, suda kaynatma mikrodalga yardımını ekstraksiyon ve ultrasonik yardımlı ekstraksiyon yöntemi olmak üzere 5 çeşit yöntem kullanılmıştır. Aktif bileşiklerin konsantrasyonları ile LC-MS/MS yöntemiyle belirlenmiştir. Ekstrakların antioksidan antienflamatuar ve 3 Clpro inhibisyon kapasiteleri kolorimetrik yöntemlerle belirlenmiştir.*

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Bulgular: *Özellikle meyan kökünün etanol özütleri antioksidan parametreler açısından değerlendirildiğinde en yüksek TEAC, FRAP ve DPPH seviyelerini göstermiştir. En güçlü 3-CLpro enzim inhibitör etkileri, 80 °C'de 6 saat bekletme, 20 dakika ultrason destekli bekletme, 40 °C'de 24 saat suda bekletme, %60 etanolde bekletme ve %80 etanolde bekletme yoluyla elde edilen meyan kökü ekstraktlarında gözlenmiştir. Meyan kökünün 3-CLpro üzerinde inhibitör bir etki gösterdiği belirlenmiştir.*

Sonuç: *Çalışmamızda, glisirizin ve glisiretinik asit gibi iyi çalışılmış biyoaktif bileşiklerin yanı sıra meyan kökündeki daha az yaygın fenolik asit ve flavonoid içeriği de incelenmiştir. Meyan kökünde analiz edilen bileşikler arasında apigenin, pelargonin, siyanidin, maleik asit, etil ferulat ve klorojenik asit en bol bulunanlarıydı. Meyan kökünün etanol ekstreleri, artan antioksidan ve anti-enflamatuar aktivitelerle ilişkili olarak daha yüksek konsantrasyonlarda fenolik ve flavonoid bileşikler göstermiştir*

Anahtar Sözcükler*: Sars-CoV-2 Glycyrrhiza glabra (meyan kökü); 3-CLpro, ekstraksiyon.*

INTRODUCTION

Coronavirus disease (COVID-19) is an infectious disease that emerged in the final days of 2019 and has become a global problem worldwide (1). Typically, it spreads through close, unprotected contact with infected individuals, often via virus-laden droplets and aerosols (2). The symptoms of the disease (such as fever, cough, shortness of breath, and pneumonia) are nonspecific and often resemble those of other upper respiratory tract infections (3). Additional symptoms include headache, fatigue, loss of smell, sore throat, increased phlegm production, runny nose, loss of appetite, and diarrhea. An infected person can develop mild to severe symptoms within 5-6 days of the incubation period. Numerous studies have shown that the mortality rate increases with age and is associated with underlying conditions, such as diabetes, hypertension, and cancer (3, 4).

The chymotrypsin-like protease (3-CLpro), which is the main protease of coronaviruses, cleaves polypeptides, including RNA-dependent RNA polymerase, helicase, ribonucleases, and other polypeptides involved in viral replication from two types of polyproteins (pp1a and pp1ab) that are crucial for viral replication (5). The replication and maturation of SARS-CoV-2 depend on the cleavage of these large polyprotein structures (3). In addition, 3-CLpro is unique to coronaviruses. Due to these important characteristics, 3CLpro has become a major drug target.

Plants have been used for the treatment of various diseases since ancient times because of their attributes of being cost-effectiveness, safety, and low toxicity, particularly in the context of infectious diseases (2, 6). Phytochemicals are important compounds in the discovery of drugs against various human diseases (7, 8). Recent research has revealed the potential of polyphenols and alkaloids to combat COVID-19 (9). Polyphenols, flavonoids, alkaloids, and other phytochemicals are abundant in plants and commonly found in the human diet. Many plant-derived products and their constituents have demonstrated significant inhibitory activities against viral infections in humans (10–12). During the SARS-CoV outbreak in 2020, treatment methods for SARS-CoV infection were explored using both traditional and modern medicine (13). Upon confirmation of clinical data, the World Health Organization (WHO) announced that the conscious use of traditional medicine, compared to modern medicine, could help reduce the mortality rate (14). In this context, studies investigating the impact of plant products on the inhibition of 3CLpro have gained significance.

Glycyrrhiza glabra, commonly known as licorice root, has been one of the most widely used medicinal plants since ancient times. Over 30 species of this plant are distributed worldwide, particularly in the Mediterranean region of Asia(15). This plant is utilized for traditional therapeutic purposes, such as treating painful swellings and coughs, and preventing the common cold. G. glabra and its bioactive phytochemicals contain properties like being anti-edematous, expectorant, anti-ulcer, anti-inflammatory, and anti-cancer (2, 16). Its widespread use in traditional medicine and scientifically proven research has turned licorice and its bioactive compounds into preferred agents for further exploration of their potential multiple health benefits (17–19).

The aim of this study was to determine the phenolic content of extracts from Glycyrrhiza glabra, a plant known for its high levels of phenolic acids and flavonoids. This study also sought to investigate the in vitro antioxidant effects of these compounds and their inhibitory effects on the 3CLpro enzyme. Furthermore, this study aimed to determine the potential role of these compounds in the treatment of SARS-CoV-2.

MATERIALS and METHODS

Chemicals

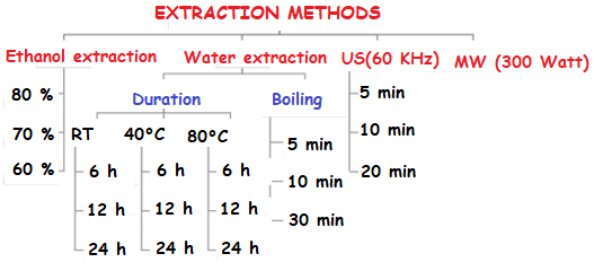
The *Glycyrrhiza glabra* plants used in this study were obtained from Dr. Henri (Izmir). The 3-CL Protease Enzyme Inhibition Kit was obtained from Bioscience (USA). All remaining chemicals and solvents were purchased from Sigma-Aldrich Co. (USA). The working principle of the 3-CLpro enzyme kit is as follows: The Unlabeled 3CL Protease Assay Kit is specifically crafted for the evaluation of 3CL Protease activity in screening and profiling applications. This assay offers a homogeneous format, eliminating the need for time-consuming washing steps. Conveniently packaged in a 96-well format, the kit includes purified untagged 3CL Protease (BPS Bioscience #100823), a fluorogenic substrate, and 3CL Protease assay buffer to facilitate 100 enzyme reactions. Additionally, the kit provides 3CL inhibitor GC376 as a control for comprehensive experimentation.

Extraction Methods

Five different methods have been used to extract active compounds to obtain various phenolic compounds (20, 21). G. glabra was prepared by subjecting 1 g of the plant sample to the extraction process at a ratio of 1 g to 10 mL.

1. **Ethanol Extraction**: Three different ethanol concentrations (60%, 70%, and 80%) were used, and plant samples were extracted in the dark at room temperature for 20 h.
2. **Water Extraction**: Extraction was performed at three different temperatures (room temperature, 40 °C, and 80 °C) and three different time durations (6, 12, and 24 h).
3. **Boiling**: Extraction was conducted in water at 100 °C for three different durations (5, 10, and 30 min).
4. **Microwave-assisted extraction**: Using water as the solvent, extraction was carried out in a microwave at 300 Watts for 15 s.
5. **Ultrasonication-Assisted Extraction Method**: Using water as the solvent, ultrasonication at a fixed frequency of 60 KHz was applied for three different durations (5, 10, and 20 min).

After each extraction, the extracts were dried and stored. On the day of use, the solution was dissolved in 10 mL water. A total of 19 samples were obtained using these methods and Scheme-1 summarizes the extraction techniques.



Scheme-1. Summary of extraction methods.

Determination of Active Ingredients and Concentrations in Plant Extracts

The determination of the concentration of active compounds in plant extracts was carried out using a Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) instrument in the EÜTF (Eskisehir Technical University Faculty) Medical Biochemistry Research Laboratory, employing methods previously used by us (22). The ultra-performance liquid chromatography (UPLC) and Mass Spectrometry (MS) conditions used in LC-MS/MS are provided in Table-1.

The concentrations of the flavonoid compounds teaflavin 3,3'-digallate, Teaflavin-3'-gallate, (-)-epigallocatechin gallate, (+)-catechin hydrate, (-)-epicatechin, (-)-epicatechin gallate, (-)-gallocatechin, (-)-gallocatechin gallate, (-)-Epigallocatechin, Amentoflavone, Quercetin, Apigenin, Genistein, and Kaempherol were determined and used as standards in the concentration measurements conducted using LC-MS/MS.

Table-1. UPLC and MS conditions of the LC-MS/MS method.

UPLC Conditions		**MS Conditions**	
System	Acquity UPLC I Class	**System**	Xevo TQD
Column	Acquity UPLC BEH C18, 2.1mm x50mm, 1.7 µm	**Ionization mode**	ES (+) ES (-)
Column Temperature	45℃	**Capilary voltage**	3.75 kV 2.52 kV
Injection volume	5 µl	**Desolvation temperature**	400℃ 400℃
Flow rate	0.3 mL/min	**Cone gas flow**	50 L/hr 0 L/hr
Mobile phase A	0.1% Formic acid in methanol	**Desolvation gas flow**	900 L/hr 900 L/hr
Mobile phase B	0.1% Formic acid in water		
Stop time	5 min		
Gradient	0-2 min A 100%; 2-2.1 min A 5%; 2.1-4 min A 100%; 4-5 min A 0%		

Determination of the Inhibitory Effect of Plant Extracts

A commercially available kit was used for this procedure. Kinetic and inhibitory measurements were performed using a fluorescent plate reader. In the case of the reaction catalyzed by protease, a method based on the increase in fluorescence emission as the substrate increased was used, and the results were monitored at 538 nm emission with a 355 nm excitation wavelength. The relative fluorescent units (RFUs) were measured and recorded using a Thermo Fluoroskan Ascent FL device.

Trolox Equivalent Antioxidant Capacity (TEAC)

The Trolox equivalent antioxidant capacity (TEAC) assay was performed according to the protocol reported by Taviano et al. in 2017. In this method, 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and potassium persulfate were diluted to final concentrations of 7 mmol/L and 4.95 mmol/L, respectively, forming a reactive phosphate buffer. Then, 190 µL of this mixture was mixed with 10 µL of plant extract. The antioxidant potential of the extract was determined by measuring the decrease in the blue-green color at 734 nm against a blank. Trolox was used as the standard for comparison and the results were expressed as Trolox equivalents (23).

Total Antioxidant (TAO)

0.1 mM DPPH (1, 1-diphenyl-2-picrylhydrazine) solution (190 µL) was mixed rapidly and thoroughly with 10 µL plant extract. The decrease in absorbance was recorded at 550 nm against a blank over a 20-minute period at 5-minute intervals using a microplate reader. Trolox was used as the standard for comparison in this assay.

Ferric Reducing Antioxidant Potential (FRAP)

In this process, the reaction mixture is prepared by mixing acetic acid buffer solution (10 mM, pH=3.6), TPTZ (2,4,6-tripyridyl-s-triazine) (10 mM), and FeCl3 (20 mM) solutions in volume ratios of 10:1:1. Subsequently, 10 µL of extract is mixed with 190 µL of this mixture. The mixture was then incubated at room temperature for 30 min, and the results were expressed as gallic acid equivalents at 620 nm using a microplate reader (24).

Hyaluronidase inhibition method

Hyaluronidase (HYA) inhibition was determined using the method described by Bralley et al. (2008). A mixture of sodium phosphate (0.2 M), sodium formate (0.1 M), and bovine serum albumin (BSA; 2 mg/10 mL) was prepared and adjusted to a pH between 6.8 and 7.2. The reaction mixture contained 20 µL the licorice root extract, 50 µL phosphate buffer, 20 µL HYA (750 units/mL, Type IV-S: bovine testis), and 50 µL Hyaluronic Acid (10 mg/mL). The mixture was then incubated at 37 °C for 30 min. A control mixture without enzymes was prepared for each extract. After incubation period, 0.1 mL of 0.8 M alkali borate was added to stop the reaction. The mixture was then placed in a water bath at 100 °C for 5 min, followed by addition of p-dimethylaminobenzaldehyde (0.5 mL). The absorbance was measured at 580 nm using water as the control. (25).

Xanthine/ Xanthine oxidase Inhibition

The study reported by Kong et al. (2000) adopted the xanthine/xanthine oxidase method (26). Xanthine was prepared by dissolving it in 0.05 M NaOH. After adding licorice extract and 80 µL of 500 µM WST-1 solution (including control samples without enzymes and extracts), the mixture was incubated at 37 °C for 15 min. Then, 0.05 U/mL of xanthine oxidase was added, and measurements were carried out at 450 nm.

Statistical Analysis and Evaluation of Results

The enzyme inhibition percentages were determined using the values obtained from samples without inhibitors (control samples) and samples containing the standard inhibitor. Enzyme activity was calculated as the change in Relative Fluorescent Units (RFUs) per minute. For multiple group comparisons, Analysis of Variance (ANOVA) was used. Correlation analysis was performed using Pearson's correlation test. Since no direct studies involving humans or animals were conducted, no ethical approval decision was required.

RESULTS

The table below shows the phenolic compound quantities in the licorice root extracts. In general, among all licorice root extracts, the phenolic compounds found at the highest concentrations were apigenin, pelargonin, cyanidin, maleic acid, ethyl ferulate, and chlorogenic acid. When looking at these compounds and enzyme inhibitions, it can be observed that ethanol extracts generally had higher phenolic acid and flavonoid contents compared to water extracts (Table-2 and Figure-1).

The extracts that exhibited the highest 3-CLpro enzyme inhibition, anti-inflammatory, and antioxidant activity levels based on the extraction method are listed in Table-2. Figure-1 shows a comparison of the concentrations of the most abundant phenolic compounds according to the extraction method. Thus, when evaluating the phenolic content, we observed that the phenolic content of licorice root extracts was in the following order, from highest to lowest: 70% EtOH > 80% EtOH > 60% EtOH.

Table-2 displays the % inhibition-HYA, % inhibition-XO, FRAP, TEAC, DPPH, and phenolic compound concentrations according to the licorice root extraction method. There was no significant difference among the groups with respect to % 3CLpro inhibition. In terms of antioxidant parameters, there was a significant difference in% HYA inhibition among the groups. There were significant differences between the groups in compounds other than delphinidin, vanillic acid, and catechin.

Table-2. Phenolic content, % inhibition for 3Cl-pro, Hyaluronidase, and Xanthine Oxidase, FRAP, TEAC, and DPPH results obtained from the extraction methods (W: Water, RT: Room Temperature, US: Ultrasound, EtOH: Ethanol, MW: Microwave, BOI: Boiling)

Compound (ng/mL)	W-RT	W-40	W-80	BOI	EtOH	US	MW
Inhibition %	71.67	74.10	82.53	73.17	85.57	78.10	85.60
HYA % inh (p=0.032*)	**61.613**	**35.869**	**95.399**	**101.221**	**103.099**	**106.667**	**96.056**
Xanthinoxidase %	0.000	18.143	28.095	22.762	0.000	0.000	0.000
FRAP	2.275	1.948	1.817	1.098	2.732	3.268	2.510
TEAC	13.666	13.828	13.852	16.203	17.046	15.228	16.798
DPPH (p=0.049*)	**74.286**	**57.143**	**84.762**	**100.000**	**100.000**	**44.762**	**60.000**
Caffeic acid (p=0.004*)	**13.634**	**40.486**	**21.932**	**19.985**	**81.330**	**49.371**	**53.939**
Ferulic acid (p=0.016*)	**320.021**	**140.132**	**146.563**	**176.594**	**67.855**	**116.445**	**198.481**
Genistein (p=0.001*)	**239.959**	**393.195**	**206.661**	**208.089**	**840.283**	**230.402**	**215.925**
Apigenin (p=0.001*)	**1100.808**	**1413.042**	**979.760**	**1010.522**	**2753.610**	**984.532**	**954.520**
Pelargonin (p=0.001*)	**4073.848**	**5995.050**	**5007.541**	**4983.269**	**25296.866**	**5450.113**	**4584.776**
Kaempferol (p=0.001*)	**173.795**	**171.987**	**135.803**	**228.911**	**289.992**	**143.111**	**166.109**
Cyanidin (p=0.001*)	**8223.025**	**8439.631**	**6684.805**	**10057.004**	**16885.383**	**7245.320**	**9516.268**
Epicatechin (p=0.028*)	**43.605**	**14.220**	**29.328**	**53.817**	**45.927**	**29.945**	**85.163**
Quercetin (p=0.001*)	**15.267**	**21.654**	**25.510**	**14.960**	**66.140**	**8.005**	**1.250**
Delphidin	50.829	235.704	123.089	147.962	378.694	139.715	39.433
Maleic acid (p=0.009*)	**2032.394**	**1384.268**	**1347.455**	**1886.286**	**452.555**	**1516.606**	**1512.052**
Salicylic acid (p=0.001*)	**15.354**	**83.585**	**103.628**	**34.180**	**216.079**	**79.138**	**28.069**
Gentisic acid (p=0.027*)	**6.511**	**54.342**	**53.563**	**51.635**	**101.431**	**31.756**	**21.685**
Procathechoic acid (p=0.012*)	**14.746**	**42.705**	**62.978**	**39.928**	**129.802**	**18.703**	**18.674**
P-Coumaric acid (p=0.001*)	**74.681**	**15.173**	**23.963**	**10.745**	**20.306**	**9.978**	**33.223**
Vanilic acid		351.568	283.873	119.308	406.041	300.751	247.168
Ethyl ferulate (p=0.002*)	**804.570**	**2002.527**	**1153.525**	**941.449**	**594.629**	**2051.967**	**1041.810**
Naringenin (p=0.0001*)	**65.648**	**244.316**	**36.028**	**50.946**	**736.492**	**31.347**	**109.918**
Catechin	7.694		29.188	40.877	54.996	6.266	20.156
Clorogenic acid (p=0.0001*)	**40.900**	**1049.533**	**1513.227**	**472.095**	**20892.444**	**1656.854**	**382.714**

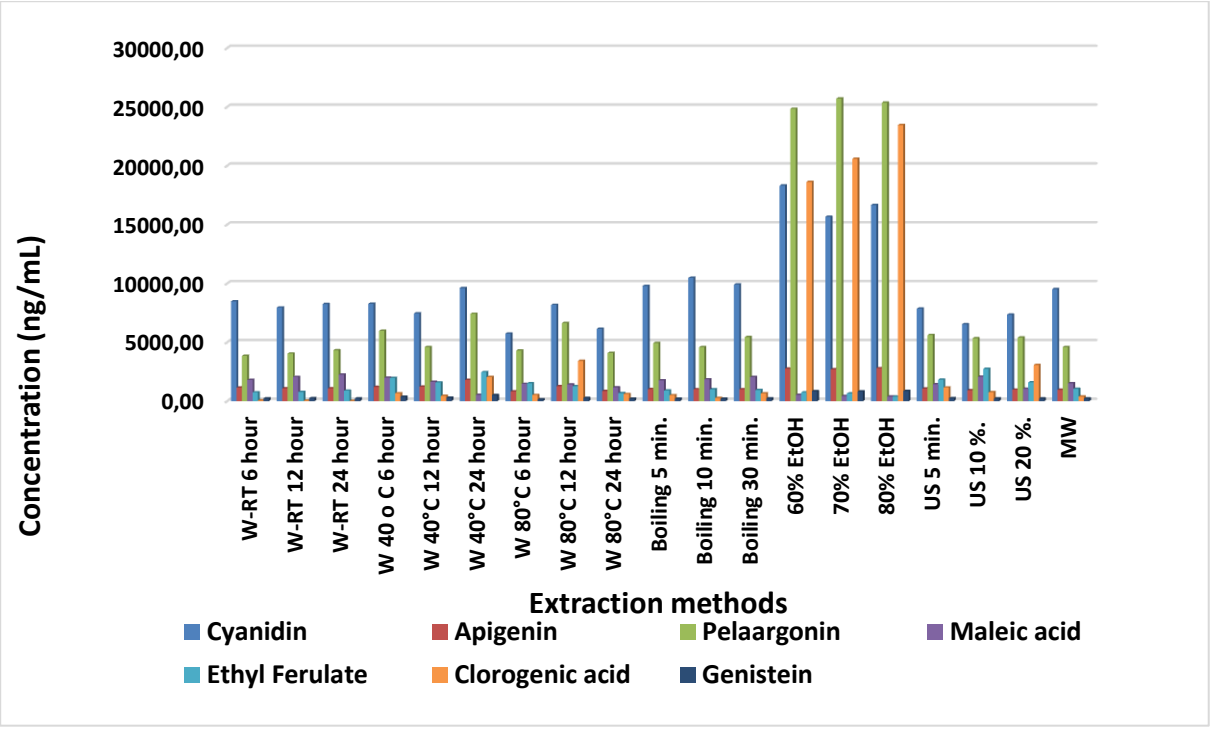


Figure-1. Comparison of the most abundant phenolic compounds according to extraction methods.

Table-3. List of extracts showing 3-Clpro inhibition, anti-inflammatory and antioxidant activity according to extraction method.

INH-3CLpro %		**FRAP**	
W-80°C -6 hour	99.4	EtOH 70%	4.39
US-20 min.	89.4	US- 5 min.	4.31
W-40°C 24 hour	87.1	W-40°C 24 hour	4.16
EtOH 60%	86.5	US-20	3.68
EtOH 80%	86.2	W-80°C 12 hour	3.61
		W-RT 24 hour	3.61
HYA inh%		**TEAC**	
Boiling-10 min.	100	EtOH 60 % and 70%	17.28 ve 17.07
Boiling-30 min.	100	W- 40°C 24 hour	17.30
EtOH 60%	100	W-80°C 12 hour	17.44
EtOH 80%	100	US-5 and MW	16.79
EtOH 70%	100	EtOH 80%	16.77
US-5-10-20 min.	100	Boiling-10 min.	16.79
Xanthinoxidase %		**DPPH**	
W-80°C 6 hour	84.285	Boiling 5-10-30 min.	100
Boiling-5 min.	59.714	EtOH 60-70-80%	100
W-40°C 12 hour	39.85	W- 80°C 6 ve 12 hour	100
W-40°C 6 hour	14.57	W- 40°C 24 hour	97.14
Boiling-30 hour	8.57	W-RT 24 hour	100

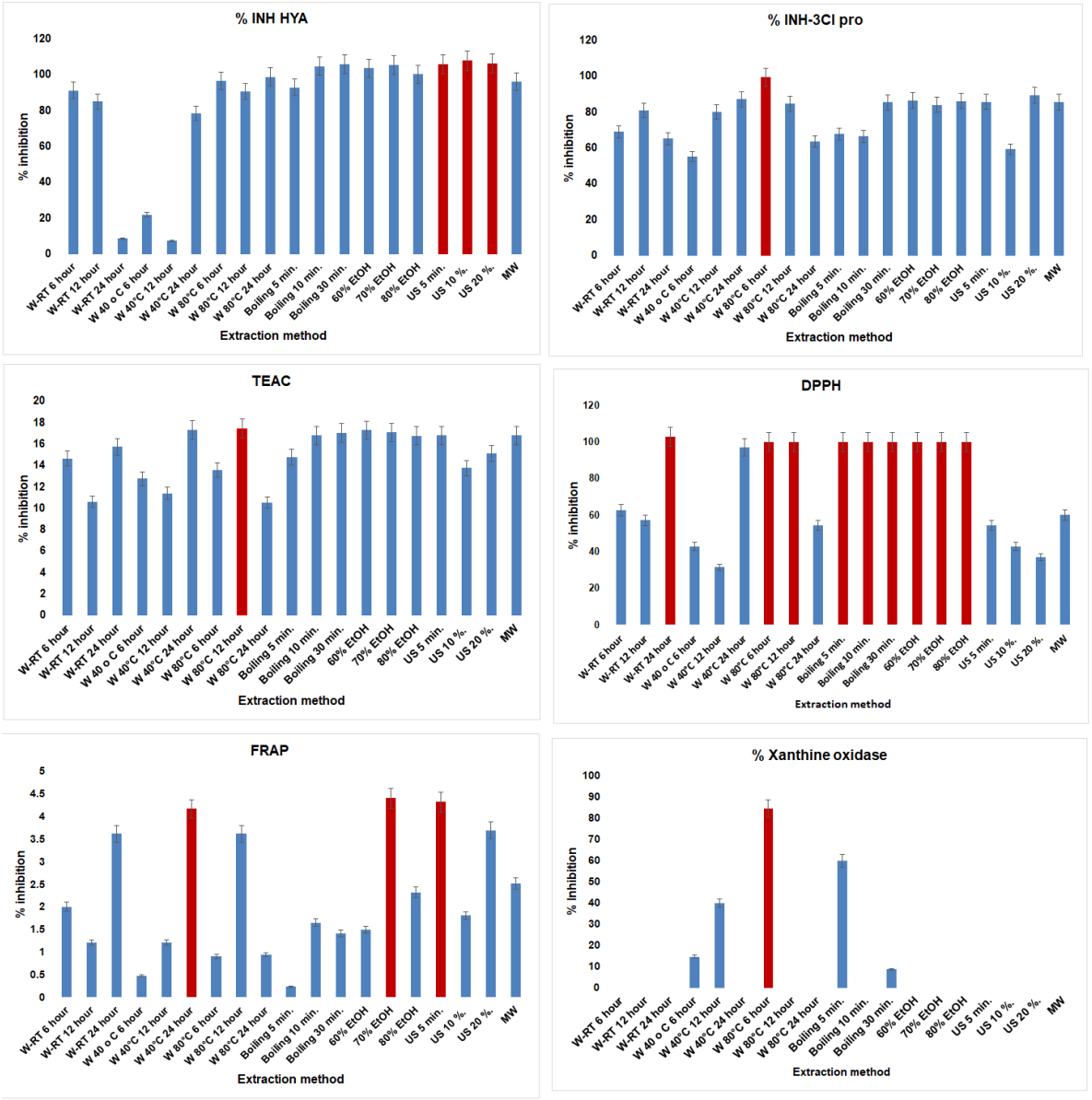


Figure-2. %inhibition, HYA %inhibition, %XO, FRAP, TEAC, DPPH levels according to Licorice Root Extraction Method. W, water; RT, room temperature; US, ultrasonication; EtOH, ethanol; MW, microwave; KAY, boiling; FRAP, ferric reducing antioxidant potential; TEAC, Trolox equivalent antioxidant capacity; HYA, hyaluronidase; DPPH, 1,1-diphenyl-2-picrylhydrazine.

Table-3 and Figure-2 shows the inhibitory effect of the extracts on the 3-CLpro enzyme, as well as their anti-inflammatory and antioxidant properties. The extracts that exhibited the highest inhibitory effect on 3-CLpro were, in order, those obtained by soaking at 80 °C for 6 h, ultrasonication for 20 min, soaking in 40 °C water for 24 h, soaking in 60% ethanol, and soaking in 80% ethanol. In terms of the parameters measuring anti-inflammatory activity, ethanol extracts yielded the highest results, except for Xanthine Oxidase activity.

The % inhibition of 3-CLpro was inversely correlated with delphinidin (p=0.016, r=-0.545), gallo-catechin (p=0.012, r=-0.562), epigallocatechin (p=0.021, r=-0.712), chlorogenic acid (p=0.046, r=-0.463), and amentoflavon (p=0.040, r=-0.475) levels. A positive correlation was observed between HYA % inhibition and delphinidin levels (p=0.006, r=0.606). No

relationship was found between the % Xanthine Oxidase and any phenolic compound concentration. FRAP positively correlated with chlorogenic acid (p=0.045, r=0.464). TEAC positively correlated with chlorogenic acid (p=0.022, r=0.523). DPPH was positively correlated with p-coumaric acid (p=0.049, r=0.669), gallocatechin (p<0.001, r=0.773), epigallocatechin (p=0.006, r=0.799), chlorogenic acid (p<0.001, r=0.759), and amentoflavon (p<0.001, r=0.792).

DISCUSSION

In this study, for the first time, the effects of licorice root on antioxidant and anti-inflammatory activities and their impact on the 3-CLpro enzyme responsible for SARS-CoV-2 replication were demonstrated. The licorice root, also known as *Glycyrrhiza glabra*, has been one of the most commonly used medicinal plants since ancient times. It contains more than 30 species distributed worldwide, particularly in the Mediterranean region of Asia (15). This plant has traditionally been used as a remedy for painful swelling, cough, and the prevention of cold and flu. *G. glabra* and its bioactive phytochemicals include properties such as anti-demulcent, expectorant, anti-ulcer, anti-inflammatory, and anti-cancer (2, 16). In the traditional medical system, it has become a preferred component to more precisely investigate the multiple health benefits offered by licorice root and its bioactive compounds, as evidenced by many previously reviewed studies (17–19). According to the literature, the therapeutic properties of licorice root extract are primarily associated with glycyrrhizin (GR) and glycyrrhetinic acid (GA) (27), which block the binding of ACE 2 to the virus spike protein, inhibit the synthesis of inflammatory factors and inflammatory mediators, and exert antiviral and antibacterial effects (28, 29). Immune cells are stimulated by multiple targets and pathways to intervene in the pathogenesis of COVID-19. Liquiritin can prevent and alleviate COVID-19 by simulating type-I I interferon. It has been suggested that licorice root can demonstrate its therapeutic advantage through multicomponent and multitarget pathways (28, 29). It has been documented that constituents of Glycyrrhiza Glabra root extract impede the growth and cellular afflictions caused by numerous unrelated RNA viruses. Licorice's water extract demonstrates antiviral activity against various viruses, including the human respiratory syncytial virus (HRSV) (30) and Enterovirus 71 in a human foreskin fibroblast cell line (31). In summary, licorice root has the potential to prevent and treat COVID-19; however, the focus has been on GR and GA for these effects, and the phenolic acids and flavonoids investigated in this study have not been the focus. The extract that showed the highest inhibition of the 3-CLpro enzyme was the water extract incubated at 80 °C for 6 h. Although ethanol extracts generally had the highest phenolic acid and flavonoid contents, water extracts exhibited higher inhibition. This may be due to the higher presence of other compounds abundant in licorice root, such as GR and GA, in the water extracts. To confirm this hypothesis, the amount of other flavonoids, especially GR and GA, should be determined in the same extracts. 3-CLpro inhibition was inversely correlated with delphinidin (p=0.016, r=-0.545), gallo-catechin (p=0.012, r=-0.562), epigallocatechin (p=0.021, r=-0.712), chlorogenic acid (p=0.046, r=-0.463), and amentoflavon (p=0.040, r=-0.475). Chlorogenic acid and delphinidin, which are found in ethanol extracts, were more abundant than in water extracts, which may explain the higher inhibitory effect of water extracts. Another reason for the higher inhibition by water extracts may be the presence of other compounds in licorice root, such as GR and GA. These compounds, along with flavonoids, could contribute to the inhibition of 3-CLpro enzyme.

Inflammation plays a significant role in epidemic and pandemic diseases, and licorice root is considered an alternative treatment (32). Inflammation is a protective measure against microbial invasion, involving action against toxins or allergens (32). However, in some cases, such as COVID-19, inflammation can become uncontrollable and cause damage to the tissues and organs. The anti-inflammatory activity and mechanism of licorice root, which resembles the action of glucocorticoids and mineralocorticoids, have been investigated by many researchers (32, 33). This action was found to be similar to that of glucocorticoids and mineralocorticoids (32). Numerous studies have indicated that licorice extract, along with its triterpenes and flavonoids constituents, demonstrates anti-inflammatory effects by inhibiting TNF, MMPs, PGE2, and free radicals (34). Flavonoids found in licorice extract exhibit significant anti-inflammatory effects in

acute inflammatory models. They notably reduce the expression of IL-1β and iNOS, as well as lower levels of NO and MDA at the inflammation site (35). In this study, extracts obtained by boiling, ethanol soaking, and ultrasound showed 100% inhibition of hyaluronidase, an anti-inflammatory enzyme. This result is consistent with the literature in terms of anti-inflammatory properties.

Glycyrrhizin/licorice extract is well-documented for its antioxidant activity, serving as a natural source of antioxidants with numerous health benefits (36). Studies highlight the excellent antioxidant properties of glycyrrhizin and other active components in licorice extract. Additionally, the high phenolic content in licorice extract has been identified as a key contributor to its potent antioxidant activity(37–40). Furthermore, glycyrrhizin and licorice extract play a role in inhibiting the generation of reactive oxygen species (ROS) by neutrophils at inflammation sites, thereby preventing tissue damage(41). When evaluating licorice root extracts in terms of antioxidant parameters, it was observed that especially ethanol extracts had the highest levels of TEAC, FRAP, and DPPH. There was a positive correlation between FRAP and chlorogenic acid ($p=0.045$, $r=0.464$), and between TEAC and chlorogenic acid ($p=0.022$, $r=0.523$). Furthermore, a positive correlation was observed between DPPH and P-coumaric acid ($p=0.049$, $r=0.669$), gallocatechin ($p<0.001$, $r=0.773$), epigallocatechin ($p=0.006$, $r=0.799$), chlorogenic acid ($p<0.001$, $r=0.759$), and amentoflavon ($p<0.001$, $r=0.792$). Chlorogenic acid is one of the six most abundant compounds in licorice root extracts studied in this research and is predominantly found in ethanol extracts, which can explain the highest levels of TEAC, FRAP, and DPPH achieved with ethanol extracts.

In conclusion, licorice root extracts that showed the highest inhibitory effect on the 3-CLpro enzyme were subjected to 6 h of soaking at 80 °C, 20 min of sonication, 24 h of soaking at 40 °C, soaking in 60% ethanol, and soaking in 80% ethanol. It was determined that licorice root exhibits an inhibitory effect on 3-CLpro. Furthermore, it was observed that the ethanol extracts of licorice root had higher concentrations of phenolic and flavonoid compounds, which correlated with higher antioxidant and anti-inflammatory activities. Glycyrrhizin, glycyrrhetinic acid, isoliquiritin, and isoflavones are the most extensively studied bioactive components of licorice root in the literature; however, this study also examined the less-documented phenolic acid and flavonoid content. Among the compounds studied in this research, the most abundant in licorice root were apigenin, pelargonin, cyanidin, maleic acid, ethyl ferulate, and chlorogenic acid.

CONCLUSION

In summary, the extracts that exhibited the highest inhibition effect on the 3-CLpro enzyme of licorice root plant were, respectively, soaking in 80°C water for 6 hours, soaking in ultrasound for 20 minutes, soaking in 40°C water for 24 hours, soaking in 60% ethanol, and soaking in 80% ethanol. The inhibitory effect on the 3-CLpro enzyme of licorice root plant has been determined. Furthermore, it has been found that the ethanol extracts of licorice root plant have higher concentrations of phenolic and flavonoid compounds, indicating greater antioxidant and anti-inflammatory activities. The most studied bioactive compounds in licorice root plant in the literature are glycyrrhizin, glycyrrhetinic acid, isoliquiritin, and isoflavones. However, this study also examined the less-explored phenolic acid and flavonoid content in licorice root plant. Among the compounds studied in this research, the most abundant compounds in licorice root plant were found to be apigenin, pelargonin, cyanidin, maleic acid, ethyl ferulate, and chlorogenic acid.

Conflict of interest: The authors have not reported any conflicts of interest.

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