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Chemical Composition, *In Vitro* Antimicrobial and Antioxidant Activities of Marine Macroalgae *Codium fragile* (Suringar) Hariot

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Abstract: Marine algae, which are the primary producers living in aquatic areas, are the subject of many studies due to their importance as they are eukaryotic and eutrophic organisms that play a crucial role in the pharmaceutical, cosmetic, food, fuel, and textile industries. Macroalgae are known in producing several macronutrients, micronutrients, and other important biologically active compounds (e.g. polyphenols, enzymes, and antibiotics) with potential pharmacological uses. In this research, we aimed to investigate the chemical composition, antimicrobial and antioxidant activities (with three assays), total phenolic (TPC) and flavonoid (TFC) contents of the methanol, ethanol, acetone, and water extracts of *Codium fragile* (Suringar) Hariot. The LC-ESI-MS/MS assessment allowed the identification of seven compounds containing gallic acid, 4-hydroxybenzaldehyde, 4-hidroxybenzoic acid, *p*-coumaric acid, salicylic acid, biochanin A, and diosgenin. TPC and TFC of the extracts were calculated as in the range of 10.34±0.13-64.67±0.02 µg GAEs/mg extract and 12.73±2.68-36.78±1.08 µg QEs/mg extract, respectively. All extracts of *C. fragile* showed antimicrobial activity against all test pathogens at different levels. The methanol, ethanol, and acetone extracts showed different levels of activity against gram-negative and gram-positive bacteria (MIC: 3.125-1.562 mg/mL). The water extract showed the highest activity in $ABTS^{\bullet+}$ (70.43±14.85%) and $DPPH^{\bullet}$ (72.61±11.44%) assays while the acetone extract exhibited the best activity in CUPRAC (absorbance: 0.60±0.15) assay. The results we obtained approved that *C. fragile* could be valued as a natural source of bioactive agents for food preservatives and in other industrial and pharmaceutical fields.

Keywords: LC-ESI-MS/MS, algae, total phenolic content, total flavonoid content, minimum inhibition concentration.

Deniz Makroalgi *Codium fragile* (Suringar) Hariot 'in Kimyasal Bileşimi, *In-Vitro* Antimikrobiyal ve Antioksidan Aktivitelerinin Analizi

Öz: Sucul alanlarda yaşayan birincil üreticiler olan deniz algleri, önemleri nedeniyle birçok araştırmaya konu olmakla birlikte ilaç, kozmetik, gıda, yakıt ve tekstil endüstrilerinde önemli rol oynayan ökaryotik ve ötrofik organizmalardır. Makroalgler, potansiyel farmakolojik kullanımları olan birkaç makro besin, mikro besin ve diğer önemli biyolojik olarak aktif bileşikler (örneğin polifenoller, enzimler ve antibiyotikler) üretmesiyle bilinmektedir. Bu araştırmada, *Codium fragile* (Suringar) Hariot 1889'un metanol, etanol, aseton ve su ekstrelerinin kimyasal bileşimi, antimikrobiyal ve antioksidan aktiviteleri (3 yöntem ile), toplam fenolik (TPC) ve flavonoid (TFC) içeriklerini araştırmayı amaçlandı. LC-ESI-MS/MS analizleri gallik asit, 4-hidroksibenzaldehit, 4-hidroksibenzoik asit, *p*-kumarik asit, salisilik asit, biokanin A ve diosgenin içeren yedi bileşiğin tanımlanmasına izin verdi. Ekstrelerin TPC ve TFC değerleri sırasıyla 10,34±0,13-64,67±0,02 µg GAEs/mg ekstre ve 12,73±2,68-36,78±1,08 µg QEs/mg ekstre olarak hesaplandı. Metanol, etanol ve aseton ekstreleri gram negatif ve gram pozitif bakterilere karşı farklı seviyelerde aktivite göstermiştir (MİK: 3.125-1.562 mg/mL). Su ekstresi $ABTS^{\bullet+}$ (%70,43±14,85) ve $DPPH^{\bullet}$ (%72,61±11,44) testlerine en yüksek aktiviteyi gösterirken, aseton ekstresi CUPRAC (absorbans: 0,60±0,15) testinde en yüksek aktiviteyi gösterdi. Elde ettiğimiz sonuçlar, *C. fragile*'in gıda koruyucuları ve diğer endüstriyel ve farmasötik alanlarda doğal bir biyoaktif madde kaynağı olarak değerlendirilebileceğini onaylamaktadır.

Anahtar kelimeler: LC-ESI-MS/MS, alg, toplam fenolik miktarı, toplam flavonoid miktarı, minimum inhibisyon konsantrasyonu.

1. Introduction

Marine life makes up more than 70% of the Earth's surface with a wide variety of life and research on this important biodiversity remains limited. Aquatic environment is recognized as a rich source of new metabolites with a variety of applications including cosmeceuticals, nutraceuticals, agrochemicals, pharmaceuticals, and other industrially related chemicals. To date, valuable bioactive compounds have been obtained from plants and terrestrial microorganisms. However, after a certain period of time, known molecules from similar organisms began to be isolated in studies. Thereupon, natural product researchers turned to obtaining new compounds from organisms found in less studied habitats (Cragg & Newman, 2013).

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Marine algae, which are the primary producers living in aquatic areas, are the subject of many studies due to their importance as they are eukaryotic and eutrophic organisms that play a crucial role in the pharmaceutical, cosmetic, food, fuel, and textile industries. Macroalgae are known for producing several macronutrients, micronutrients, and other important biologically active compounds (e.g. polyphenols, enzymes, and antibiotics) with potential pharmacological uses (Aşıkkutlu & Okudan, 2021; Gümüş et al., 2021). Macroalgae are known for producing several macronutrients (lipids, proteins, carbohydrates, fibers, and the like), micronutrients (minerals and vitamins), and other important biologically active compounds (e.g. polyphenols, enzymes, and antibiotics) with potential pharmacological uses (Arguelles et al., 2019a; Ortiz et al., 2006; Muraguri et al., 2016).

Some studies reported that marine environments are a rich source of new bioactive metabolites and most of them are much different than those obtained from soil-derived organisms (Cragg & Newman 2013). As known, a lot of recent researches were focused on important bioactive compounds identified in macroalgae and described the range of biochemical and pharmacological activities. It is known that bioactive secondary metabolites synthesized by macroalgae have antimicrobial activity (Liao et al., 2003).

Many marine macroalgae have both primary and secondary metabolites with novel structures and are biologically active. Macroalgae especially contain reactive antioxidant molecules, secondary metabolites, comprised of carotenoids (fucoxanthin, astaxanthin, carotene (alfa,beta), catechins (e.g., epigallocatechin, catechin), and mycosporine-like amino acids (mycosporine-glycine), gallate, tocopherols, and eckol phlorotannins (e.g., phloroglucinol) (Kolsi et al., 2017). Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are synthetic antioxidants that are preferred to be added to many foods to reduce degradation caused by oxidation. Nevertheless, due to some of their toxic safety problems, naturally available antioxidants are preferred more (Witschi & Lock, 1978).

The presence of phenolic structures in macroalgae was first reported by Crato (1893). Phenolic compounds are the name given to a group of compounds containing hydroxyl (-OH) on an aromatic hydrocarbon ring. Polyphenols found in macroalgae are tannins, catechins, flavonoids, phlorotannins, and some phenolic acids. These phenolic compounds have such significant pharmaceutical properties as antiproliferative, antibacterial, antidiabetic, antikoagulan, antiviral, antihelmintik, anti-inflammatory, anti-HIV, antioxidant, antiparasitic, antiallergic, and anti-tumoral ones. In addition, the correlation between the total phenolic content and antioxidant activity of macroalgal extract is highly affected by the extraction method. Mostly 70% acetone has been reported to be more effective than water in extracting polyphenolic compounds. (Kadam et al., 2019)

Codium fragile (Suringar) Hariot is a marine agae belonging to the Codiaceae (Chlorophyta) family. This macroalgae is also known as dark green marine algae, ranges from 15 to 45 cm. Moreover, it consists of branching cylindrical segments (Fig. 1). The genus *Codium* is represented by about 125-130 species widely distributed in all seas of the world except the polar seas and is mostly found in subtropical and temperate seas (Keskinkaya et al., 2020).

Figure 1. Morphological properties of *C. fragile*. a) underwater view of *C. fragile*, b) air-dried form of *C. fragile*, c) dust form *C. fragile*.

In this study, we were interested in the beneficial properties of "*Codium fragile* (Suringar) Hariot 1889", a dark green macroalgae collected from the Güzelyalı-Çanakkale region. The aims of this investigation are to analyze the chemical composition by LC-ESI-MS/MS systems of *C. fragile* collected from Güzelyalı-Çanakkale (Türkiye) and to investigate antimicrobial (using minimum inhibition concentration method) and antioxidant three *in vitro* assays DPPH$^{\bullet}$ (2,2′-diphenyl-1-picrylhydrazyl) radical scavenging activity ABTS$^{\bullet+}$ (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) and CUPRAC (cupric reducing antioxidant capacity) activities.

These assays may provide some important information about the possible antimicrobial capacity and antioxidant mechanism with chemical composition of all extracts. For relevant extraction, we used methanol, ethanol, water, and acetone solvents. Total phenolic content (TPC) and total flavonoid content (TFC) were determined to evaluate the nature of the antioxidants present in these extracts. We believe that this general screening experiment will provide a basis for future characterization and isolation studies to select the most suitable macroalgae species and to evaluate the suitability of these extracts as natural antioxidants for pharmaceutical applications in various real drug and food systems.

2. Material and Methods

2.1. Algae Samples Collection

Samples of *Codium fragile* was collected from a sampling as deep as 0-5 m station in Güzelyalı-Karanlık Liman, 15 km far from Çanakkale. The position of Güzelyalı-Karanlık Liman is centered on 40°14'27.03"N - 26°32'29.74"E as follows in Fig. 2.

Codium fragile belong to the genus Chlorophyta. The systematic classification of the algae types used in our study is as follows in Table 1 (AlgaeBase).

The collected macroalgae samples were washed with ambient water to remove foreign substances. Then, macroalgae samples were placed in sterile polyethylene

bags and brought to the cold chain laboratory. They were washed with distilled water in the hydrobiology laboratory to remove epiphytic creatures and necrotic particles from the samples.

In drying process, the algae were placed in an oven set at 40°C to prevent the phytochemical compounds from being damaged and pre-drying was carried out by keeping it for 17 h. Marine macroalgae were dried correctly, and and pulverized using a homogenizer, stored at room temperature until extraction.

Table 1. The systematic classification of *C. fragile* (AlgaeBase).

Species	Kingdom	Phylum	Subphylum	Class	Order	Family	Genus
Codium fragile	Plantae	Chlorophyta	*Chlorophytina*	*Ulvophyceae*	*Bryopsidales*	*Codiaceae*	*Codium*

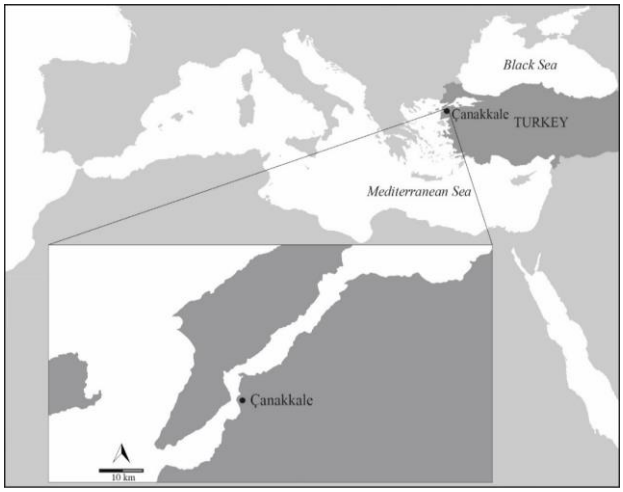

Figure 2. Map of algae taken from Güzelyalı/Çanakkale.

2.2. Preparation of Algae Extracts

The soxhlet extraction method was applied to the grinded marine macroalgae samples to obtain extracts. Macroalgae samples (10 g) were extracted with various solvents according to the increasing polarity: acetone, methanol, ethanol, and water for 6 h by using the soxhlet apparatus. The methanol, ethanol, and acetone were evaporated under a vacuum by an evaporator to obtain all the extracts. The water was lyophilized to get the water extract by using a freeze-drier. All macroalgal extracts were stored at +4°C until analysis.

2.3. Chemical Composition

2.3.1. Preparation of *C. fragile* and standard solutions

10 mg of algae extracts were prepared at a concentration of 2 mL in methanol and the solution was diluted to 2 mg/mL with 50% methanol in grade water. Subsequently, the solution was filtered through 0.45 µm filters and transferred into vials prior to LC-ESI-MS/MS analysis.

2.3.2. LC-ESI-MS/MS instrumentation conditions

An Agilent Technologies 1260 Infinity II liquid chromatography System combined to a 6460 Triple Quad mass spectrometer were used for quantitative and quantitative analysis of 56 phytochemical compounds. Poroshell 120 EC-C18 (100 mm × 4.6 mm I.D., 2.7 µm) column was used for the chromatographic separation of the compounds. Mobile phase flow rate, column temperature conditions, and different mobile phase additives such as formic acid, ammonium acetate, and acetic acid were applied together with acetonitrile, purified water, and methanol mobile phases to achieve the most ideal separation and ionization of the compounds. Thus, in chromatographic separation, mobile phases of 0.1% formic acid and 5mM ammonium format in water A mobile phase and 0.1% formic acid and 5mM ammonium format in methanol B mobile phase were used. Moreover, using a flow rate of 0.4 mL/min, a gradient program of 15% for 1-12 min, 50% for 12-30 min, 90% for 30-32 min and 10% for 32-35 min was applied in the B mobile phase, respectively. The column temperature was maintained at 40°C and the injection volume was 4.0 µL (Yılmaz, 2022).

An electrospray ionization (ESI) source operating in both negative and positive ionization modes was used to determine the mass to ion ratio (m/z) of the compounds. The ESI Source parameters were set at capillary voltage to 4000 V, nebulizing gas (N_2) flow to 11 L/min, nebulizer pressure to 15 psi and gas temperature to 300°C to ensure ideal ionization of all compounds and achieve the ideal peak intensity. The product and precursor ions, their collision energies and fragmentor voltage were determined the measurement as multiple reaction monitoring (MRM) (Yırtıcı et al., 2022).

2.3.3. Total phenolic (TPC) and total flavonoid contents (TFC)

TPC of the marine macroalgae extracts was measured according to the Folin Ciocalteu method (Slinkard & Singleton, 1977). Results were calculated using the following equation obtained from the standard gallic acid graph:

Absorbance=0.0104 [gallic acid (µg)] - 0.0263, (r^2, 0.9924)

TFC of the marine macroalgae extracts was measured according to the aluminum nitrate method (Park et al., 1997). Results were calculated using the following equation obtained from the standard quercetin graph:

Absorbance=0.0158 [quercetin (µg)] - 0.0306 (r^2, 0.9993)

2.4. Bioactivity Assays

2.4.1. Antimicrobial activity

The broth microdilution method reported by Alsenani et al. (2020) was used to determine the antimicrobial activities of the marine macroalgae extracts. The antimicrobial test was performed by determining minimum inhibitory concentration (MIC) values of different marine macroalgae extracts (0.0061-6.25 mg/mL) against fungus, gram-positive, and gram-negative bacterial strains. In addition, we used negative growth control DMSO (100%) and positive growth control contained gentamisin (0.1 mg/mL). The lowest concentration values for bacterial inhibition were calculated and reported as a MIC.

2.4.2. Antioxidant activity

Antioxidant activities of the marine macroalgae extracts were tested using DPPH• (2,2′-diphenyl-1-picrylhydrazyl) free radical scavenging, $ABTS^{\bullet+}$ (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) cation radical scavenging, and CUPRAC (Cupric reducing antioxidant capacity) activity assays (Çayan et al., 2019). Ascorbic acid, BHT, and BHA were used as standards. The IC_{50} value (50% inhibition activity) was calculated using the graph plotted between the percentage of antioxidant activity (inhibition%) and the concentration (µg/mL) of the extracts. The $A_{0.50}$ value (concentration having 0.50 absorbance) was calculated using the graph plotted between the absorbance and the concentration (µg/mL) of the extracts. Results were given as IC_{50} values and inhibition percentage (%) at 400 µg/mL concentration for radical scavenging assays; $A_{0.50}$ values and absorbance at 400 µg/mL concentration CUPRAC assay.

2.5. Statistical Analysis

All data obtained in the section of antioxidant activity analyses of this study were analyzed using SPSS 25.0 for Windows (Statistical Package for Social Sciencies) and G-Power programs. Descriptive analyses were made for continuous variables and arithmetic mean ± standard deviation values of the variables were given. Whether the data were normally distributed or not was evaluated using the Shapiro-Wilk test. As a result of the tests, it was determined that the data did not comply with the normal distribution in case of $p<0.05$. The homogeneity of the variances was examined with the Levene test. As a result of the test, homogeneity of variance could not be achieved. Since the parametric test conditions could not be met in the study, the comparison of the numerical data between independent multiple groups was analyzed using the Kruskal Wallis H test and the comparisons between the two groups were analyzed using the Mann Whitney U test. The power of the study was determined as 76% with a Type 1 error of 5% and an effect size of d=0.5. The statistical power criterion, which is aimed to be found above 80% in studies, was approached in the study in question (Cohen, 1962; 1977). While interpreting the analyses results, the error was kept at the level of 0.05; thus, the decisions were made at the 95% confidence level.

3. Results and Discussion

3.1. Chemical Composition

Chemical composition was quantitatively determined in the extract of *Codium fragile* as seen in Table 2 and Fig. 3 A. Fifty-six compounds as phenolics, flavonoids, and other compounds were quantitatively analyzed in the extracts using the LC-ESI-MS/MS system and also chromatograms of the standard compounds and the extracts are given in Fig. 3. Diosgenin was identified as major compound in the methanol (4.96 µg/g), ethanol (108.1 µg/g), and water (62.45 µg/g) extracts while biochanin A was found as the main phenolic compound in the acetone extract (98.57 µg/g). Diosgenin is a valuable secondary metabolite belonging to the class of steroidal saponins that has an important place in the pharmaceutical industry (Hernández-Vázqueza et al., 2020). Studies have shown that diosgenin has antioxidant, anti-cancer, anti-aging, cardioprotective, contraceptive, antiviral, antimicrobial, antifungal, and insecticidal activities (Chaudhary et al., 2018). Biochanin A is an isoflavone and have various effects consisting of antioxidant, anti-inflammatory, estrogen-like, and glucose and lipid metabolism modulatory, cancer preventive, neuroprotective, and drug interaction effects (Yu et al., 2019).

There is a limited number of studies about chemical composition of *Codium* species in the literature. Cinnamoyl glucose, sinapine, 5-(3',5'-dihydroxyphenyl)-γ-valerolactone, dihydrobiochanin A, scopoletin, rosmanol, carnosol, deoxyschisandrin, and carnosic acid were identified in *Codium* sp. by LC-ESI-QTOF-MS/MS (Zhong et al., 2020). The main phenolic compounds of the ethanol and water extracts of 16 algae species (*Fucus serratus, F. vesiculosus, F. distichus, F. spiralis, Sargassum muticum, Saccharina latissima, Laminaria digitata, Dictyota dichotoma, Enteromorpha intestinalis, Ulva lactuca, Palmaria palmata, Porphyra purpurea, Chondrus crispus, Mastocarpus stellatus, Polysiphonia fucoides, Gracilaria vermiculophylla*) were identifed by HPLC in the study of Farvin & Jacobsen (2013). Similar to the results we obtained, the amount of phenolic compounds in the water extracts was reported lower than the ethanol extracts. In addition, all the water extracts were found to have gallic acid and trace levels of chlorogenic acid. LC-MS/MS analysis showed highest phloroglucinol (69.86±5.25 mg/kg), fucoxanthin (1.45±0.22 mg/kg), and gallic acid in *Sargassum wightii*; highest quercetin (0.07±0.00 mg/kg) and ferulic acid (0.21±0.04 mg/kg) in *Ulva rigida;* and the highest vanillin (0.39±0.01 mg/kg) in *Gracilaria edulis* (Kumar et al., 2020). It has been reported that these variations between chemical compositions are related to many factors such as macroalgae species, sources, extraction and purification techniques, and storage conditions (Cotas et al., 2020).

3.2. Total Phenolic (TPC) and Total Flavonoid Contents (TFC)

TPC and TFC results of the methanol, ethanol, acetone, and water extracts of *Codium fragile* were shown in Fig. 4. TPC of the extracts ranged between 10.34±0.13 and 64.67±0.02 µg GAEs/mg extract. The highest concentration of TPC was found in the acetone extract (64.67±0.02 µg GAEs/mg extract). TFC of the extracts ranged between 12.73±2.68 and 36.78±1.08 µg QEs/mg extract. The highest concentration of TFC was found in the ethanol extract (36.78±1.08 µg QEs/mg extract).

Previously TPC of the ethanol (80%), methanol (70%), hot water, and cold water extracts of *Codium fragile* were recorded in the range of 0.99±0.1-17.27±0.06 µg GAE mg^{-1} sample (Heffernan et al., 2015). TPC of the ethanol extract and ethyl acetate and water fractions of *C. fragile* were found as 2.202±0.103, 22.381±0.206 and 0.298±0.103 mg GAE g^{-1} DW, respectively (Surget et al., 2017). TPC (~45, 50, 60 mg GAE g^{-1} extract, respectively) and TFC (~30, 40, 50 mg QE g^{-1} extract, respectively) of the hexane, ethyl acetate, and methanol extracts of *C. fragile* were calculated by Kolsi et al. (2017). There are similarities and differences between the results and the literature. In general, the number of phenolic compounds is affected by nature, extraction procedure used, sample particle size, storage conditions and time as well as the assay used to determine them and the presence of interfering substances in extracts. Quantitative isolation of the phenolic compounds is very difficult due to their size and molecular weight, structural similarity, and propensity to react with other compounds.

Due to different extraction conditions and result expression, these differences between the studies could be explained (Mekinic et al., 2019; Schoenwaelder, 2002).

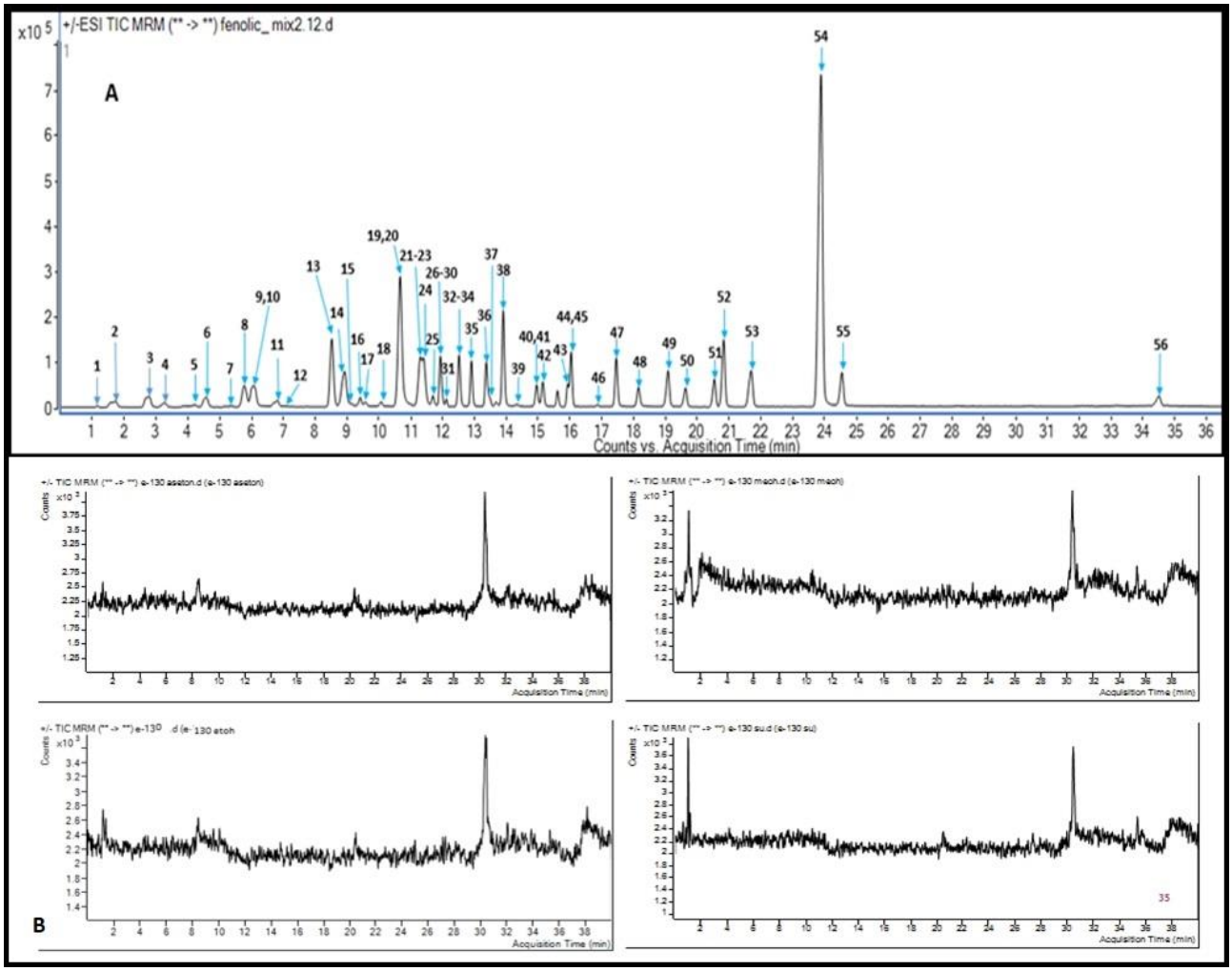

Figure 3. LC–ESI–MS/MS MRM chromatograms of (A) the standard compounds 1-Shikimic acid, 2-Gallic acid, 3-Protocatechuic acid, 4-Gentisic acid, 5-Catechin, 6-4-Hydroxybenzoic acid, 7-Chlorogenic acid, 8- 4-Hydroxybenzaldehyde, 9-Vanillic acid, 10-Caffeic acid, 11-Epicatechin, 12-Syringic acid 13-*p*-coumaric acid, 14-Salicylic acid, 15-Taxifolin, 16-Polydatine, 17-*trans*-ferulic acid, 18-Sinapic acid, 19-Quercimeritrin, 20-Coumarin, 21-Scutellarin 22-*o*-coumaric acid, 23-Cynarin, 24-Protocatechuic ethyl ester, 25-Hyperocide, 26-Quercetin-3-glucoside, 27-Isoquercitrin, 28-Resveratrol, 29-Naringin, 30-Rutin, 31-Rosmarinic acid, 32-Quercetin-3-D-xyloside, 33-Kaempferol-3-glucoside, 34-Hesperidine, 35-Neohesperidin, 36-Fisetin, 37-Oleuropein, 38-Baicalin, 39-*trans*-cinnamic acid, 40-Ellagic acid, 41-Quercetin, 42-Naringenin, 43-Silibinin, 44-Hesperetin, 45-Morin, 46-Kaempferol, 47-Tamarixetin, 48-Baicalein, 49-7-Hydroxyflavone, 50-6-Hydroxyflavone, 51-Biochanin A, 52-Chrysin, 53-Flavone, 54-5-Hydroxyflavone, 55-6,2,4-Trimetoxyflavone and 56-Diosgenin. (B) compounds in the various extracts (acetone, methanol, ethanol, water) of *Codium fragile.*

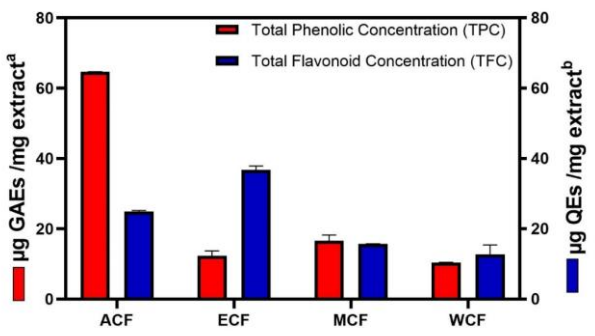

Figure 4. TPC and TFC of the extracts of *Codium fragile.* [a:] gallic acid equivalent [b:] quercetin equivalent, ACF: CF Acetone Extract, ECF: CF Ethanol Extract, MCF: CF Methanol Extract, WCF: CF Water Extract

3.3. Antimicrobial Activity

The global epidemic of bacterial resistance against existing antibiotics has led to the discovery of antibacterial agents from natural sources. Over millions of years, the evolution of bacteria and antibacterial biomolecules may provide the potential to overcome resistant strains. In this context, studies on the discovery of new agents from terrestrial and marine resources are gaining momentum (Shannon & Abu-Ghannam, 2016). There are reported studies that describe the antibacterial capability (derived from secondary and primary metabolites) of macroalgae against medically important pathogenic bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus, Aeromonas hydrophila, Clostridium perfringens*, *Escherichia coli*, *Klebsiella pneumoniae,* and *Enterobacter aerogenes* (Arguelles et al., 2019b; Liu et al., 2017; Ibtissam et al., 2009; Lima-Filho et al., 2002).

Antimicrobial activities of the extracts of *Codium fragile* were investigated using the microdilution method and results were given in Table 3. Among the studied extracts, the methanol, ethanol, and acetone extracts showed different levels of activity against gram-negative and gram-positive bacteria (MIC: 3.125-1.562 mg/mL). The water extract displayed the weakest antimicrobial activity against tested pathogens *Escherichia coli* (MIC: 6.25 mg/mL) and *Klebsiella pneumoniae* (MIC: 6.25 mg/mL). The ethanol and acetone extracts were more active than the other extracts; thus, it can be thought that these antimicrobial activities of the extracts may be due to phytochemicals such as phenolics, flavonoids, and diosgenin.

Table 2. Analysis of chemical composition (µg/g) in *Codium fragile* extracts by using LC-ESI-MS/MS.

Phenolic compounds	R_t (min)	LOD (µg/L)	LOQ (µg/L)	R^2	Methanol	Ethanol	Acetone	Water
Gallic acid	1.74	4.8	15.25	0.999	ND	0.20	ND	ND
4-hydroxybenzaldehyde	5.77	8.78	26.7	0.998	ND	0.35	12.99	ND
4-hidroxybenzoic acid	4.54	19.25	54.12	0.999	ND	ND	ND	0.79
p-coumaric acid	8.50	2.25	7.8	0.999	0.27	ND	ND	ND
Salicylic acid	8.89	15.94	47.84	0.999	ND	ND	10.35	ND
Biochanin A	20.59	2.45	7.81	0.999	ND	99.07	98.57	ND
Diosgenin	34.51	3.13	8.19	0.999	4.96	108.1	2.21	62.45

R_t, retention time, LOD and LOQ: limit of detection and limit of quantification.

ND: not detected.

Table 3. Minimum inhibitory concentration (MIC) of *Codium fragile* extracts.

Test Microorganism	MIC (mg/mL)					
	Water	Methanol	Ethanol	Acetone	Gentamycin (0.1 mg/mL)	DMSO
E. coli	6.25	3.125	3.125	NA*	<0.02	12.5%
P. aeruginosa	NA*	1.562	NA*	NA*	<0.02	12.5%
K. pneumoniae	6.25	NA*	NA*	NA*	0.78	12.5%
S. aureus	NA*	3.125	3.125	1.562	<0.02	25%
S. enteritidis	NA*	3.125	3.125	3.125	0.04	12.5%
S. lutea	NA*	1.562	1.562	1.562	<0.02	12.5%
B. cereus	NA*	NA*	NA*	1.562	<0.02	12.5%
C. albicans	NA*	NA*	NA*	1.562	<0.02	12.5%

*NA: not active

The species belonging to the *Codium* genus are the least studied in terms of their biological activities and antimicrobial activity among all Chlorophyceae. *C. fragile* is among the macroalgae that attracts attention due to its invasive nature and its use in biomedical applications (Kim et al., 2013). Frikha et al. (2011) evaluated the antibacterial activities of *C. dichotomum*, *C. fragile*, *C. bursa* and *C. tomentosum* against pathogenic bacteria (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 700603 and *Enterococcus ATCC 700603*) and reported that all methanol extracts of algae showed significant activity against *S. aureus* but no significant activity was observed in *C. bursa*. The ethanol extracts of *C. bursa* were used against *E. coli* and *Staphylococcus* and all algal extracts showed significant antibacterial activity against the bacteria studied (Frikha et al., 2011). The reports of this study show parallelism with the results of our study in terms of the activity of the ethanol and methanol extracts. Jun et al. (2018) evaluated MIC of 11 different macroalgae against tested pathogens and they reported that *C. fragile* have no growth inhibitory effect against any pathogens. In the other study, the methanol extract of *C. intricatum* showed an extended spectrum of inhibitory activity against gram-positive drug-resistant bacterium, methicillin-resistant *S. aureus* (MRSA) with MIC of 250.00 µg/mL. It was moderately active against penicillin-acylase producing *Bacillus cereus* with MIC of 250 µg/mL. However, no inhibitory effect was observed among the tested gram-negative bacterial pathogens (Arguelles, 2020). According to this reported study, the methanol extract of *Codium* sp. did not show any activity against gram-negative bacteria but in our study the methanol extract was effective against both gram-negative (*E. coli, Salmonella enteritidis* - MIC*:* 3.125 mg/mL, *Pseudomonas aeruginosa* - MIC: 1.562 mg/mL) and gram-positive bacteria (*S. aureus* - MIC*:* 3.125 mg/mL, *Sarcina lutea* - MIC: 1.562 mg/mL). Antibacterial activity is thought to be affected by algae reproductive status and seasonality (Ibtissam et al., 2009).

Another point is that all of the extracts prepared with various solvents showed the strongest inhibition effects (MIC: 1.562 mg/mL) against gram positive bacteria. This situation can be explained as the susceptibility of gram positive bacteria to the algal extracts was more than those of gram negative bacteria. Many authors reported similar observations (Demirel et al., 2009; Ibtissam et al., 2009). The more susceptibility of gram-positive bacteria to the algal extracts was due to the differences in their cell wall

structure and their composition (Taskin et al., 2007). In gram-negative bacteria, the outer membrane acts as a barrier to many environmental substances including antibiotics (Tortora et al., 2001). The presence of thick murine layer in the cell wall also prevents the entry of the inhibitors (Kandhasamy & Arunachalam, 2008).

The remarkable differences and similar points between the results obtained in our study and in previous studies may be due to several factors. First of all, this can be because of the intraspecific variability in the production of secondary metabolites, occasionally related to seasonal variations and these variations are seen in other published reports (Lima-Filho et al., 2002; Moreau et al., 1988). Secondly, these variations could be related to the different solubility actions of secondary metabolites that could be affected by the species' geographical and seasonal distribution and there may also be differences in the capability of the extraction protocols to recover the active metabolites and differences in the assay methods that would result in different susceptibilities of the target strains (Gonzalez et al., 2001; Perez at al., 1990).

Even the antibacterial capacity of macroalgae extracts changes depending on different parameters such as type of macroalgae, solvent, extraction method, extract concentration, and type of microorganism (Rajasulochana et al., 2009).

3.4. Antioxidant Activity

Since antioxidants have different action mechanisms, more than one method is preferred to determine the antioxidant activity rather than a single method. Antioxidant activities of *Codium fragile* extracts were screened using ABTS$^{\bullet+}$ cation radical scavenging activity, DPPH$^{\bullet}$ free radical scavenging, and CUPRAC activity assays. The results were summarized in Table 4-10.

Table 4. Antioxidant activity of *Codium fragile* extracts.

	Antioxidant Activity					
	DPPH$^{\bullet}$ assay		ABTS$^{\bullet+}$ assay		CUPRAC assay	
Extracts	Inhibition (%)[a]	IC_{50} (µg /mL)[b]	Inhibition (%)[a]	IC_{50} (µg/mL)[b]	Absorbance[c]	$A_{0.50}$ (µg/mL)[d]
Methanol	2.03±1.67	>400	11.54±8.64	>400	0.18±0.04	>400
Ethanol	1.90±0.40	>400	20.88±12.85	>400	0.25±0.11	>400
Acetone	1.34±0.74	>400	16.72±10.54	>400	0.60±6.880.15	>400
Water	72.61±11.44	>400	70.43±14.85	>400	0.20±0.07	>400
Standards						
Ascorbic acid	79.71±9.45	6.68±0.22	80.92±9.29	5.24±0.18	1.96±1.43	44.06±0.09
BHT	62.16±27.87	23.90±0.14	72.03±19.16	12.75±0.63	1.49±1.23	28.21±0.01
BHA	64.49±26.49	22.80±0.59	75.53±19.69	12.05±0.97	1.37±1.16	26.54±0.02

[a]: Inhibition % of 400 µg/mL concentration of the extracts.

[b]: IC_{50} values are given as a mean ±SD of three parallel measurements.

[c]: Absorbance of 400 µg/mL concentration of the extracts

[d]: $A_{0.50}$ values are given as a mean ±SD of three parallel measurements.

Table 5. Intergroup statistical results for ABTS$^{\bullet+}$ activity of *Codium fragile* extracts

	ABTS$^{\bullet+}$ assay			
Extracts	n	Inhibition (%)	K-S	*p*-değeri*
Methanol	24	11.54± 8.64	55.061	0.000*
Ethanol	21	20.88±12.85		
Acetone	24	16.72±10.54		
Water*	24	70.43±14.85		

*Multiple comparisons evaluated with Kruskal Wallis H, pairwise comparisons with Mann Whitney U test. α=0.05

Table 6. Kruskal-Wallis test results of ABTS$^{\bullet+}$ activity differences between *Codium fragile* extracts according to their solvents.

		ABTS$^{\bullet+}$ assay			
Extracts	Standards	n	Inhibition (%)	K-S	*p*-değeri*
Methanol	Ascorbic acid	24	80.92±9.29	56.655	0.000*
	BHT	24	72.03±19.16		
	BHA	24	75.53±19.69		
Ethanol	Ascorbic acid	24	80.92±9.29	47.687	0.000*
	BHT	24	72.03± 19.16		
	BHA	24	75.53±19.69		

Table 6. (Continued)

		ABTS•+ assay			
Extracts	Standards	n	Inhibition (%)	K-S	*p*-değeri*
Acetone	Ascorbic acid	24	80.92±9.29	54.899	0.000*
	BHT	24	72.03±19.16		
	BHA	24	75.53±19.69		
Water	Ascorbic acid	24	80.92±9.29	5.906	0.116
	BHT	24	72.03± 9.16		
	BHA	24	75.53±19.69		

*Multiple comparisons evaluated with Kruskal Wallis H, pairwise comparisons with Mann Whitney U test. α=0.05

Table 7. Intergroup statistical results for DPPH• activity of *Codium fragile* extracts

	DPPH• assay			
Extracts	n	Inhibition (%)	K-S	*p*-değeri*
Methanol	24	2.03± 1.67	57.090	0.000*
Ethanol	15	1.90± 0.40		
Acetone	24	1.34±0.74		
Water*	21	72.61± 11.44		

*Multiple comparisons evaluated with Kruskal Wallis H, pairwise comparisons with Mann Whitney U test. α=0.05

Table 8. Kruskal-Wallis test results of DPPH• activity differences between *Codium fragile* extracts according to their solvents

		DPPH• assay			
Extracts	Standards	n	Inhibition (%)	K-S	*p*-değeri*
Methanol	Ascorbic acid	24	79.71± 9.45	55.017	0.000*
	BHT	24	62.16±27.87		
	BHA	24	64.49±26.49		
Ethanol	Ascorbic acid	24	79.71± 9.45	38.732	0.000*
	BHT	24	62.16±27.87		
	BHA	24	64.49± 26.49		
Acetone	Ascorbic acid	24	79.71±9.45	52.025	0.000*
	BHT	24	62.16± 27.87		
	BHA	24	64.49±26.49		
Water	Ascorbic acid	24	79.71±9.45	5.701	0.232
	BHT	24	62.16±27.87		
	BHA	24	64.49±26.49		

*Multiple comparisons evaluated with Kruskal Wallis H, pairwise comparisons with Mann Whitney U test. α=0.05

Table 9. Intergroup statistical results for CUPRAC activity of *Codium fragile* extracts

	CUPRAC assay			
Extracts	n	Absorbance (%)	K-S	*p*-değeri*
Methanol	12	0.18±0.04	27.121	0.000*
Ethanol	12	0.25±0.11		
Acetone*	12	0.60±0.15		
Water	12	0.20±0.07		

*Multiple comparisons evaluated with Kruskal Wallis H, pairwise comparisons with Mann Whitney U test. α=0.05

Table 10. Kruskal-Wallis test results of CUPRAC activity differences between *Codium fragile* extracts according to their solvents.

		CUPRAC assay			
Extracts	Standards	n	Absorbance (%)	K-S	*p*-değeri*
Methanol	Ascorbic acid	24	1.96±1.43	29.775	0.000*
	BHT	24	1.49±1.23		

Table 10. (Continued)

		CUPRAC assay			
Extracts	Standards	n	Absorbance (%)	K-S	*p*-değeri*
	BHA	24	1.37±1.16		
Ethanol	Ascorbic acid	24	1.96±1.43	21.395	0.000*
	BHT	24	1.49±1.23		
	BHA	24	1.37±1.16		
Acetone	Ascorbic acid	24	1.96±1.43	5.221	0.156
	BHT	24	1.49±1.23		
	BHA	24	1.37±1.16		
Water	Ascorbic acid	24	1.96±1.43	23.099	0.000*
	BHT	24	1.49±1.23		
	BHA	24	1.37±1.16		

*Multiple comparisons evaluated with Kruskal Wallis H, pairwise comparisons with Mann Whitney U test. α=0.05

The water extract showed the highest activity in ABTS$^{\bullet+}$ (70.43±14.85%) and DPPH$^{\bullet}$ (72.61±11.44%) assays. There are statistically significant differences in ABTS$^{\bullet+}$ activity between *Codium fragile* extracts (p<0.00). After paired comparisons, it was determined that the water extract had higher antioxidant activity than the other extracts. The activities of methanol, ethanol, and acetone extracts were statistically similar (Table 5). For ABTS$^{\bullet+}$ activity, no statistical difference was found between the water extract and standards (ascorbic acid, BHT, and BHA). Therefore, antioxidant activity of the water extract was found to be similar to the standards (p=0.116>0.05) (Table 6). There were statistically significant differences in DPPH$^{\bullet}$ activity between the extracts (p<0.00). After paired comparisons, it was determined that the water extract had higher antioxidant activity than the other extracts. The activities of the methanol, ethanol, and acetone extracts were statistically similar (Table 7). For DPPH$^{\bullet}$ activity, no statistical difference was found between the water extracts and standards (ascorbic acid, BHT, and BHA). Therefore, antioxidant activity of the water extract was found to be similar to the standards (p=0.232>0.05) (Table 8). In the CUPRAC assay, the best activity was recorded in the acetone extract with the absorbance value of 0.60±0.15. There were statistically significant differences in CUPRAC activity between the extracts (p<0.00). After paired comparisons, it was determined that the acetone extract had higher antioxidant activity than the other extracts. The activities of the methanol, ethanol, and acetone extracts were statistically similar (Table 9). For CUPRAC activity, no statistical difference was found between the acetone extract and standards (ascorbic acid, BHT, and BHA). Therefore, the antioxidant activity of the acetone extract was found to be similar to the standards (p=0.156>0.05) (Table 10). According to the obtained results, the best antioxidant activity was found in the water extract in DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays. It is well known that marine algae are rich in sulfated polysaccharides and glucans. These polysaccharides have been proven to act as anti-inflammatory, anticoagulant, antioxidant, antiviral, anti-tumor, anti-obesity, and antimicrobial agents *in vitro* and *in vivo* (Garcia-Vaquero et al., 2019). As a result, the highest antioxidant activity of the water extract can be related with the synergic effects of the polysaccharides and the identified compounds.

Antioxidant activities of various extracts of *Codium fragile* were reported in earlier studies. Antioxidant activity of the hexane, dichloromethane, and methanol extracts of *C. fragile* was investigated by β-carotene bleaching and hydroxyl radical scavenging assays with inhibition values of ~50-80% and ~50-70% respectively at 40 mg/mL concentration (Koz et al., 2009). Surget et al. (2017) studied antioxidant activity of the ethanol extract and ethyl acetate and water fractions of *C. fragile* according to DPPH$^{\bullet}$ and reducing power assays. Among the studied extract and fractions, the ethyl acetate fractions were found as the best active in DPPH$^{\bullet}$ (IC_{50}: 0.303±0.002 g/L) and reducing power (IC_{50}: 5.478±0.891 g/L) assays. In the study of Heffernan et al. (2015), antioxidant activity of ethanol (80%), methanol (70%), hot water, and cold water extracts of *C. fragile* was tested by DPPH$^{\bullet}$ (IC_{50}: 0.13±0.01-0.56±0.01 mg/mL) and FRAP (0.94±0.03-32.70±0.10 µg Tr equivalents mg^{-1} sample) assays. Our results agree with previous studies.

4. Conclusion

As a result, in this study, antimicrobial and antioxidant activities of the methanol, ethanol, acetone, and water extracts of *Codium fragile* marine macroalgae species were screened with TPC and TFC. Chemical compounds thought to be responsible for these bioactivities were confirmed by LC-ESI-MS/MS analysis. Our results showed that the four algal extracts have a strong wealth in phenolic compounds and flavonoids have a very interesting antioxidant status allowing that should be considered as an important source of phenolic compounds that could be used as food preservatives and in other industrial and pharmaceutical fields. Moreover, this study concludes that *C. fragile* represents an alternative natural source of polyphenols and other bioactive compounds for the development and production of natural antioxidants and novel antibiotics. Further studies should be conducted to identify the structure and elucidate the mechanism of action of different biologically active metabolite present in the macroalgae extracts that showed promising antimicrobial and antioxidant activities.

Considering both the terrestrial and marine resources that Türkiye has, we come across a great treasure to be explored. As new isolation studies are carried out, we

think that these studies will provide success in many areas and pave the way for important investments.

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