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Investigation of antioxidant properties of olive leave extracts from Hatay by different extraction methods

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Abstract: In this study, it was aimed to compare the antioxidant properties of the extracts obtained from the extraction of olive leaves in Hatay province prepared with different solvents. For this purpose, olive leaves were extracted using pure methyl alcohol with soxhlet and also by maceration using 60% ethanol, 70% methanol, 90% acetone (v/v) and distilled water. Total Flavonoid Content (TFC), Total Phenolic Content (TPC), Ferric Antioxidant Reducing Power (FRAP), DPPH radical scavenging avctivity, ABTS Cation Radical Scavenging activity and total sulfydryl groups by Ellman methods were used to determine the antioxidant properties of the extracts. As a result of the study, the highest TFC values per g leaf and g extract were obtained for 70% methanol maceration and soxhlet-methanol extract, respectively, while the highest TPC observed per leaf and g extract were determined in the extracts obtained with 90% acetone. In the FRAP method, the extract obtained with 60% Ethanol showed the highest activity per g leaf and g extract. Extracts obtained with Soxhlet showed the highest activity for both ABTS activity and Ellman method. In the DPPH method, the lowest EC50 value was determined in the extract obtained using 70% methanol, and it was determined that the extracts obtained with water showed the lowest performance in all antioxidant activity methods.

Keywords: Olive leaves, Extraction, Antioxidant activity, Soxhlet, Maceration

1. Introduction

It is stated that there are an estimated 500 million olive trees in the world and 90% of them are located in the Mediterranean. It has been reported that there are more than 13 million fruit bearing and approximately 3 million non-fruiting olive trees throughout the province of Hatay (2020-2021 Yield report).

Olive leaf extract is obtained from olive tree leaves by physical and chemical methods. In recent clinical studies, It has been shown that the olive leaf extract is effective in many conditions such as viral, bacterial and fungal infections, HIV-AIDS, chronic fatigue, hepatitis B, pneumonia, tuberculosis, influenza, common cold, meningitis, shingles, Epstein-barr virus, encephalitis, gonorrhea, severe diarrhea, ear, tooth, urinary tract and surgical infections (Page 2002). It has been reported that the olive leaf has been used in the treatment of malaria and febrile diseases for many years, and the oleuropein in the leaf is an antioxidant and has an antimicrobial effect against viruses, bacteria, molds, fungi and parasites (Benavente-Garcia et al. 2000). At the same time, it has been reported that olive leaves, which have biological value, are a natural, safe and inexpensive alternative antioxidant source and even have the feature of extending the shelf life in foods due to their antimicrobial effects and inhibition of lipid oxidation (Jemai et al. 2009; Boudhrioua et al. 2009; Bouaziz et al. 2010). Utilizing the phenolic compounds found in olive leaves at the highest level can only be achieved by maintaining an effective extraction and solubility properties (Mourtzinos et al. 2007).

Atoms or molecules with one or more unpaired electrons are called free radicals. Such substances are highly reactive because they have unpaired electrons. Free radicals which play an important role in biological systems gain or lose electrons through any interaction. Therefore, they can be positive, negative or neutral (Gönenç et al. 2002). Antioxidants are substances that significantly inhibit or delay oxidation when present in food or in low concentrations in the body (Aeschbach et al. 1995). Food manufacturers use synthetic food preservative antioxidants to prevent food spoilage and preserve the nutritional value of food. Antioxidants are of interest to biochemists and health professionals because they protect the body against damage caused by reactive oxygen species and degenerative diseases.

The market for medicinal and aromatic plants is estimated to be approximately US$14 billion per year and will reach more than US$5 trillion by 2050. About 3000 medicinal and aromatic plant species are traded worldwide, 2000 of which belong to European countries such as Switzerland, Germany and France (Thakur and Kumar, 2021). while India has an important position with 7000 medicinal and aromatic plant species (Prasathkumar et al. 2021). Olive leaves show antioxidant properties due to the functional bioactive compounds in their structure. Thanks to this feature, it has been reported that olive leaf extracts can be used to extend the quality and shelf life of foods (Erbay and Icier 2010; Abaza et al. 2011).

Although few literatures have been reported to reveal the different biological effects of olives regarding its anti-inflammatory, anti-allergic, antibacterial, antimycotic, immunoregulatory, antidiabetic and hypolipemic (Omar 2010) effects; studies of *in vivo* or *in vitro* effects of olive leaves that remain after the olive harvest and destroyed by burning are unsufficient. In addition, variables such as the method used in the extraction process, shredding, drying, temperature and organic solvent type also change the amount of antioxidant substances (Tsimidou and Papoti 2010).

Antioxidants are molecules that prevent or slow down the oxidation of biological macromolecules in living things. The role of antioxidants is to neutralize oxidative molecules via hydrogen atom or electron transfer. Therefore, antioxidants are generally substances with reducing, radical scavenging, oxidizing properties such as polyphenols or thiols (Adwas et al. 2019). To determine antioxidant power, besides determining the amount of phenolic, flavonoid and thiol substances, which are important in showing effectiveness as an antioxidant system, it is also essential to analyze their activities such as radical scavenging, radical quenching and reduction. The antioxidant activity properties of the olive leaves have become one of the remarkable issues. Studies in this regard have gained momentum recently. The antioxidant properties of olive leaves extracts varies according to the type of olive, the agricultural process applied, the region where it is grown and the harvest time as well as the extraction method and the type of solvent used. Antioxidant activity determination methods are important in terms of getting an idea for this purpose. Relative antioxidant capacity is an important antioxidant activity determination method for comparing the antioxidant capacity of different foods, food components measured by two or more chemical analyzes (Amensour et.al. 2010).

The TEAC analysis uses ABTS' heavily colored cation radicals to test the antioxidants' ability to quench radicals. TEAC analysis is widely used for testing antioxidant capacity in food samples (Re et al.1999). With the ABTS method, a large number of samples can be scanned in a short time.

The ferric reducing antioxidant power (FRAP) assay is a typical electron transfer-based method that measures the reduction of ferric ion (Fe^{3+})-ligand complex to the intensely blue-colored ferrous (Fe^{2+}) complex by antioxidants in an acidic medium (Kumar S. 2018).

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol. The biggest advantage of reagent interact with all substances in the mixture, including the weakest antioxidants, and it can react with lipophilic as well as hydrophilic antioxidants. Therefore, it is an easy, reliable and fast method (Kedare and Singh 2011).

In this study, it was aimed to compare the effects of solvents used during extraction on antioxidant activity in olive leaf extracts. For this, five different methods which were reported in the literature were selected and leaf extraction was carried out by applying the soxlet or maceration, and then the total flavonoid content (TFC), total phenolic content (TPC) of these extracts and antioxidant activities were determined by FRAP, DPPH, ABTS, Ellman methods.

2. Materials and Method

Ethanol, methanol, acetone, sodium carbonate, aluminum chloride, $K_3[Fe(CN)_6]$ were obtained from Merck; 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Folin – Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trolox were purchased from Sigma. All other chemicals used are analytical grade. Spectrophotometric measurements were performed with a Perkin Elmer UV-Visible Lambda spectrophotometer.

2.1. Leaves sampling and extraction

The olive leaves were collected from the land in Altınözü district of Hatay (36°08'62"N and 36°30'17"E) (Fig. 1).

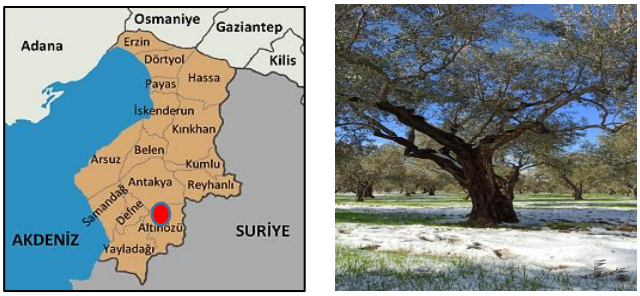


Fig. 1 Sampling area

The leaves were collected in November of 2021, and on the same day they were sorted and washed with water and then cleaned with pure water. The filtered leaves were dried at room temperature for 7 days and then ground. In the extraction step, 60 ml of solvent per 1 gram was used for all methods. For the extraction processes, 5 methods were selected, one of which is soxlet and the other four are maceration. For Soxhlet, methanol was used as a solvent, and for maceration, 90% acetone, 60% ethanol, 70% methanol and pure water were used as solvents. Maceration was carried out in a shaker incubator at 40 °C for 4 hours; In the soxhalet extraction, 3 siphons were made. After extraction, the extracts were filtered and

stored at +4°C until use.Extraction procedure is given in Fig.2.

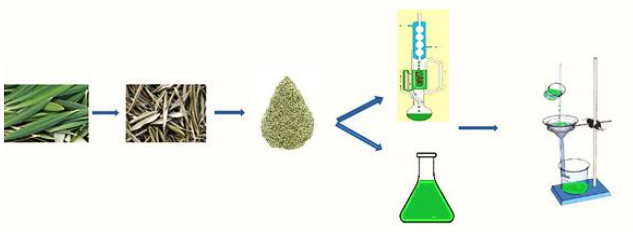


Fig. 2 Extraction of the olive lea

2.2. Antioxidant activities

Extracts were used after 4 fold dilution for TFC, TPC and FRAP assay while 60 fold diluted extracts were used for ABTS, DPPH and Ellman assays. Experimental studies were carried out in 5 parallels.

2.2.1. Total flavonoid content (TFC)

The flavonoid content (TFC) was determined by the aluminum chloride method using quercetin as the reference compound (Kumaran and Karunakaran 2006). After the extracts obtained, 0.5 ml was taken and after adding 2 ml of water and 150 µl of 5% $NaNO_2$ aqueous solution, respectively, it was waited for 5 minutes. Then, 150 µl of 10% $AlCl_3$ aqueous solution was added and waited for 1 min. Finally, after the addition of 1 ml of 1 M NaOH, the absorbance was read at 510 nm. The TFC value of the extracts was calculated as mg quercetin per gram leaf or per gram extract.

2.2.2. Total phenolic content (TPC)

The total phenolic content of the olive leaf extract was determined by the Folin Ciocalteau method (Gutfinger 1981) according to the gallic acid standard. 2.5 ml of water and 0.25 ml of Folin reagent were added to 0.5 ml of the extracts, and 750 µl of 20% Na_2CO_3 was added 5 minutes later. After 2 hours in dark, absorbances were read at 750 nm and TPC values were calculated as mg gallic acid equivalent per gram leaf or g extract.

2.2.3. Ferric reducing antioxidant power-FRAP assay

In this method, $Fe(CN)_6^{3-}$ is reduced and the formed $Fe(CN)_6^{4-}$ reacts with Fe^{+3} to form the $Fe[Fe(CN)_6]^-$ complex, which gives maximum absorption at 700 nm (Hue et al. 2012). As a result of the reaction, a dark blue complex is formed, and the higher the absorbance of the complex, the higher the reducing power. 0.4 ml of 0.2 M phosphate buffer at pH 6.6, 0.4 ml of 1% of $K_3Fe(CN)_6$ were added to 1 ml of extract, respectively, and it was kept at 50 °C for 20 minutes, 1 ml of TCA was added and centrifuged for 10 minutes. 1.5 ml of supernatant was taken from the supernatant and 1.5 ml of distilled water and 0.3 ml of $FeCl_3$ were added to it, 10 minutes after the absorbance was read at 593 nm. Trolox was used as a standard and results were calculated as mg trolox equivalent reducing power per g leaf and gram extract.

2.2.4. Trolox equivalent antioxidant capacity (TEAC)-ABTS assay

This method is based on the scavenging of ABTS radical cation by antioxidants. ABTS radical is formed by mixing 7 mM ABTS and 2.45 mM $K_2S_2O_8$ in a 2:1 ratio and keeping it in the dark for 16 hours. The prepared radical is diluted with ethanol until its absorbance is fixed at 0.7 at 734 nm. Then, after adding 3 ml of the radical ready to use in the experiment to the test tubes, 5 minutes after adding 100 µl of the extracts, the absorbance (A) was read at 734 nm. Inhibition percentage was calculated using Eq.1

$$\text{Inhibition \%} = [(Ao-A)/Ao].100 \qquad \text{Eq.1}$$

TEAC was calculated as mg trolox equivalent per gram of leaf or gram of extract.

2.2.5. DPPH assay

This method is based on measuring the scavenging effects of antioxidants with a stable free radical, 1,1-diphenyl-2-picrylhydrazine (DPPH) (Molyneux 2004). DPPH radical scavenging capacity analysis methods are a frequently used method to measure the antioxidant capacity of natural extracts (Mot et al. 2011). The purple colored DPPH radical solution gives maximum absorption at 517 nm. In this method, which is based on radical reduction by adding antioxidant to ethanol or methanolic DPPH solution, a decrease in absorbance is observed at 517 nm. In the method, after adding 2 ml of DPPH solution to each of the test tubes, 5 different concentrations of the extract were added and incubated at room temperature for 30 minutes. At the end of the period, absorbance was read at 517 nm. Extract concentrations versus % inhibition values were plotted and the concentration (EC50) values corresponding to 50% inhibition were calculated and compared with the standard antioxidants BHA and BHT.

2.2.6. Determination of total sulfhydryl groups by Ellman assay

In this method, thiol groups, which have antioxidant properties, interact with DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)), also known as Ellman's reagent, and an equivalent amount of 5-thio-2-nitrobenzoic acid is formed. The absorbance of the yellow product formed is measured at 412 nm and the results are evaluated by using the standard curve drawn for cysteine (Hansen et al. 2007).

Ellman's reagent was prepared as 0.1M DTNB in 0.1 M pH 8 phosphate buffer containing 1 mM EDTA. After adding 250 microliters of extract to 2.5 ml of the reagent, it was kept at room temperature and in the dark for 15 minutes, then the absorbance was read at 412 nm. Results were calculated as mg cysteine equivalents per gram leaf or gram extract.

3. Results and Discussion

Extracts obtained with soxleth-methanol, 90% acetone, 70% methanol, 60% ethanol and distilled water were dried

by lyophilization and 0.196, 0.194, 0.151, 0.176 and 0.275 g of extract were obtained per gram leaf, respectively.

3.1. Total flavonoid contents (TFC) of olive leaf extracts

TFC of the extracts obtained as a result of the applied extraction methods is given in Fig. 3 in terms of mg quercetin per gram leaf and gram extract (mg QE/g).

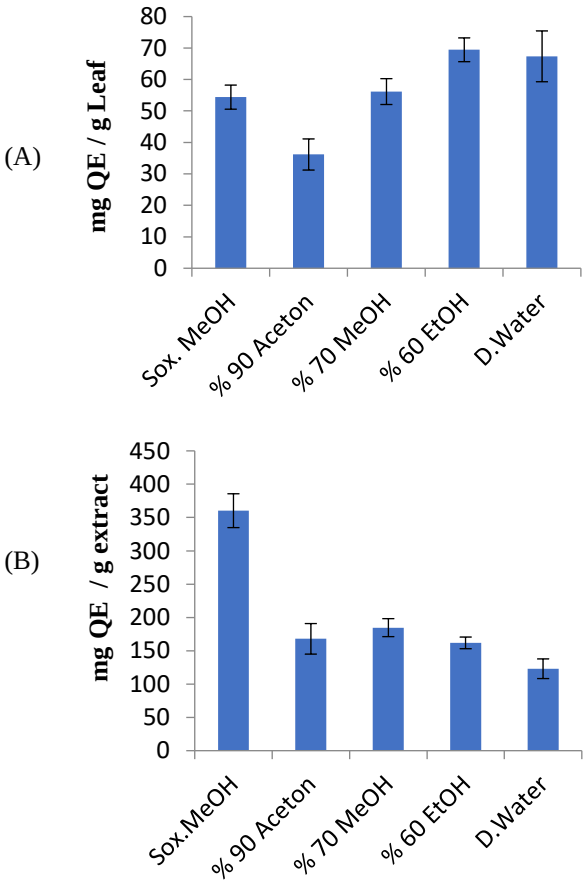


Fig.3 TFC values per gram leaf (A) and gram extracts as mg quercetin equvialent

As seen in Figure 3, the highest TFC value per g leaf was 70% methanol as 69.44 mg quercetin; 360.21 mg of quercetin per g extract was obtained in soxhlet-methanol extraction. The TFC values of the extracts made with water were the lowest both per g leaf and g extract.
In order to determine the effect of age (Çetinkaya et al. 2016). periodically investigated the TFC values in the leaves of the 9 and 65 years old trees of Kilis Yağlık cultivar. The highest and lowest TFC values were determined as 87.24 mg QE/g in August and 65.42 mg QE/g in October, respectively, in old tree leaves. However, in young tree leaves, the highest and lowest values were determined as 84.27 mg QE/g in April and 61.05 mg QE/g in October, respectively (Khizrieva et al. 2021) obtained olive leaf extract by water-alcohol and also by subcritical water at 120, 180 and 220 °C; and they reported TFC values as 33.0, 25.4, 29.7 and 65.2 mg rutin equvalient/g, respectively.

3.2. Total phenolic contents (TPC) of olive leaf extacts

The TPC values of the extracts obtained in the study were determined by the Folin method and calculated as mg gallic acid equivalent (mg GAE) per gram leaf and gram extract, and the results were presented in Fig. 4.
As seen in Fig. 4, the highest TPC values per g leaf and g extract were determined as 25.58 mg GAE in 90% acetone and as 128.86 mg GAE in Soxhlet-methanol extracts, respectively. It was determined that the lowest TPC values was the extract obtained by using water 11.31 mg GAE/g leaf and 38.47 mg GAE/g extract.

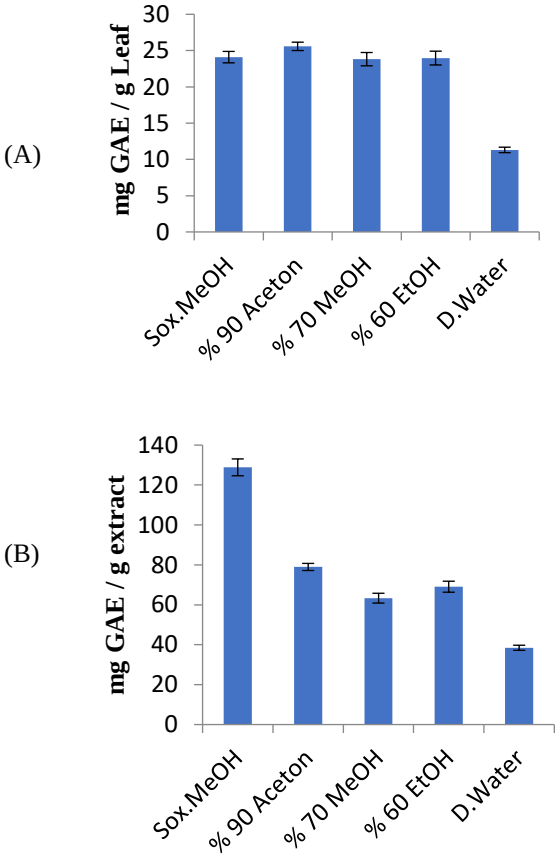


Fig. 4 TPC values per gram of leaf (A) and per gram of extracts (B) as Gallic acid equivalent (GAE) amount

(Abaza et al. 2011) used 80% methanol, 70% ethanol, 80% acetone and deionized water as solvents to investigate the effect of solvent type on the phenolic substance and antioxidant activity values of Chetoui olive leaf extracts. According to the results obtained, they reported that the highest value of the total amount of phenolic substance in the extract prepared using 80% acetone was 24.09 ± 0.73 mg GAE /g dry matter and (Khizrieva et al. 2021). Obtained olive leaf extract by water-alcohol and also by subcritical water at 120, 180 and 220 °C; and they reported TPC values as 42.6, 32.7, 41.8 and 70.4 mg GAE/g, respectively.

3.3. Ferric reducing antioxidant power (FRAP) of olive leaf extracts

FRAP values of leaf extracts were given in Fig. 5. as mg trolox equvialent (TE). As can be seen in Fig. 5(A), the FRAP values calculated per gram leaf were the smallest in water extract (11.31 mg TE), while they were quite close to each other, approximately 25 mg TE in those of other methods. When the FRAP values per gram extract given in Fig. 5(B) were examined, it was observed that the highest reducing power belonged to methanol-soxlet extract (128.86) and the lowest reducing power belonged to water extract (38.47 mg TE) (Cho et al. 2020). investigated the effect of TPC, antioxidant and antimicrobial activity by extracting olive leaves with ethanol, methanol, 50%, 70% and 90% (v/v) aqueous solutions of acetone, as well as deionized water.

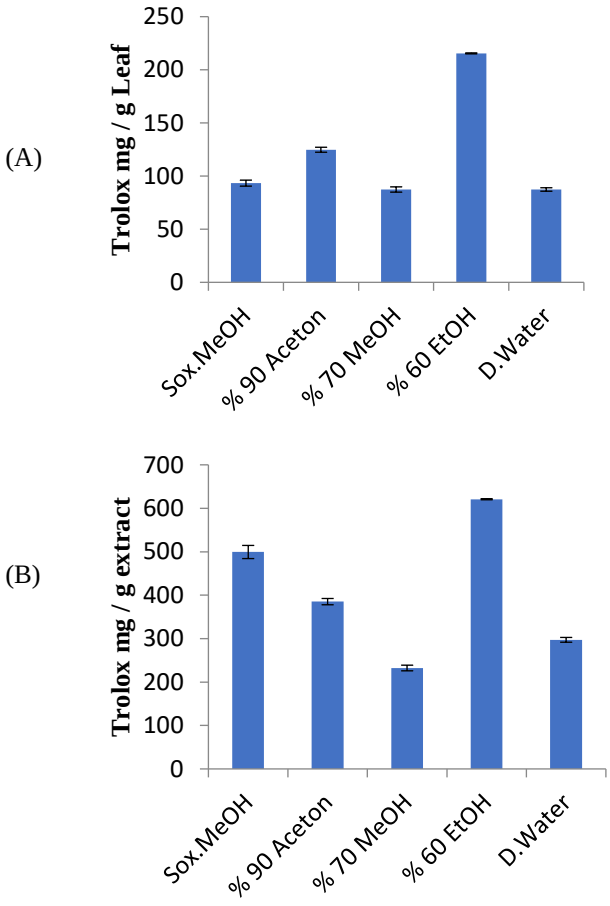


Fig. 5 FRAP values per gram of leaf (A) and per gram of extracts (B) as trolox equivalent amount

As a result of the study, when the extracts were analyzed by HPLC for oleuropein, hydroxytyrosol and tyrosol contents, they reported that the highest extraction efficiency was obtained as 20.41% when using 90% methanol solution. The highest TPC values were obtained as 232 and 231 mg GAE/100 g for 90% methanol and 90% ethanol, respectively. Also, They determined that the highest values in DPPH, FRAP and Fe^{2+}-chelating activity and antioxidant activity were obtained when 90% methanol was used.

3.4. Trolox equivalent antioxidant capacity (TEAC)-ABTS of olive leaf extracts

In this method, the effect of quenching ABTS cation radical of the extracts was investigated, using trolox as a standard and the results were given as trolox equivalent (TEAC) (Fig.6).

As seen in Figures 6(A) and 6(B), the highest activity per g leaf and g extract was 18.82 TEAC mg/g leaf and 60.01 TEAC mg/g extract value in the extract obtained from methanol-soxhlet extraction, whereas the lowest activity was 3.34 TEAC mg/g leaf and 10.31 TEAC mg/g extract and the extract obtained by using water. Sevim and Tuncay (2012) determined the ABTS antioxidant activity in Ayvalık and Memecik olive leaves as 825.38-1056.16 µmol TE/100 g (Martin-Garcia et al. 2022). investigated ABTS scavenging activity and reported between 26.92 and 35.7 mg TE/g extract in 7 different olive leaf extracts cultivated in Spain.

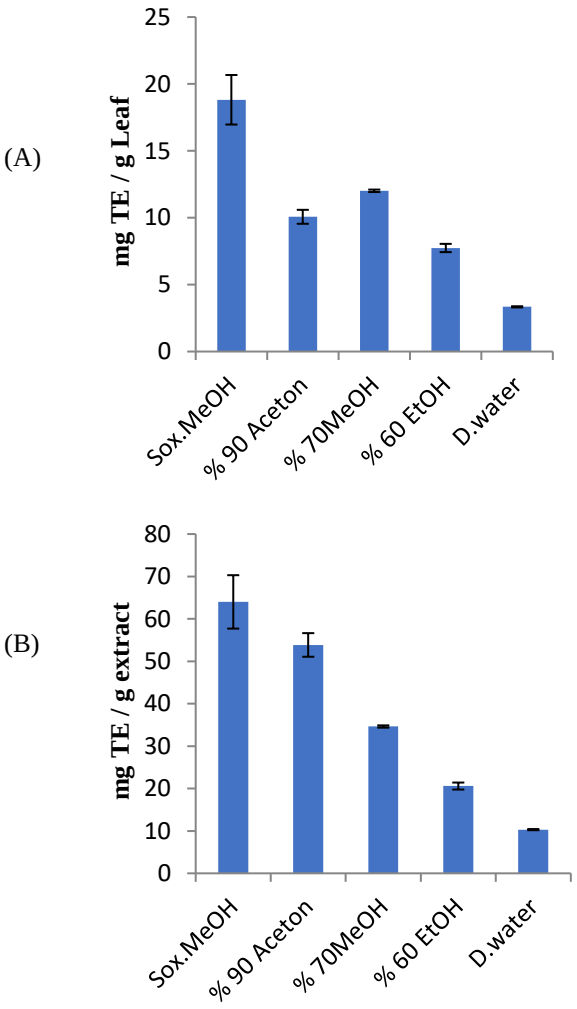


Fig. 6 ABTS scavenging activities per gram of leaf (A) and per gram of extracts (B) as trolox equivalent amount

3.5.DPPH radical scavenging activities of olive leaf extracts

The DPPH radical scavenging activities of the leaf extracts obtained per gram of leaf and gram of extract as EC_{50} are given in Table 1. The results are compared with BHA and BHT. As seen in Table 1, Soxhlet-Methanol extract showed incredibly higher DPPH scavenging activity. In particular, the activity per gram extract was quite close to the BHA standard in soxlet-methanol extraction; and also , in 70% methanol and water extracts, DPPH scavenging activity as high as BHT was observed.

Table 1 EC_{50} values of extracts and BHA and BHT standards obained by DPPH assay

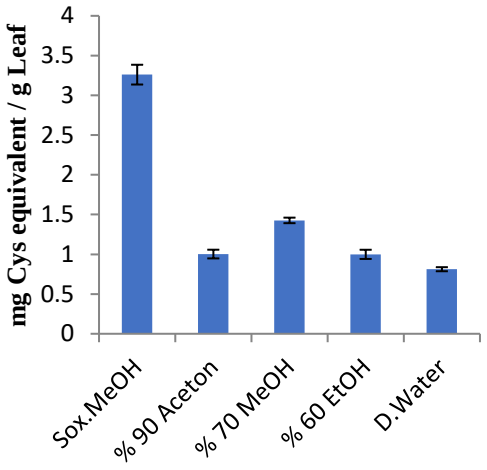
	EC_{50} (µg /ml)	
	leaf	**Extract**
Sox.MeOH	10.34±7.49	2.03±1.47
% 90 Acetone	200.00±0.00	38.80±0.00
% 70 MeOH	150.17±0.29	22.67±0.04
% 60 EtOH	177.27±14.29	31.20±2.52
D.Water	275.90±15.38	20.87±4.23
BHA	1.00±0.35	
BHT	22.92±2.89	

Martin-Garcia et al. (2022) determined DPPH scavenging activity ranging from 33.03 to 46.8 mg TE/g extract in 7 different olive leaf extracts collected in Spain.

3.6. Total sulfhydryl groups of olive leaf extracts by Ellman assay

In this method, total free sulfhydryl groups were determined spectrophotometrically by the Ellman method and the results were calculated as mg cysteine equivalents. The variation of the amounts of free sulfhydryl groups per gram leaf and gram extract is given in Figure 6. As seen in Figure 6(A), the highest values per gram leaf and gram extract were observed as 3.26 mg CysE/g leaf and 10.06 mg CyS E/g extract, respectively, in the samples obtained with soxlet-methanol. It was also observed that the results obtained with other methods are close to each other.

(A)



(B)

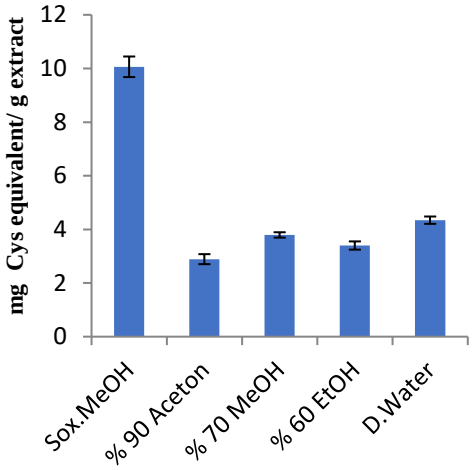


Fig.7 Total sulfydryl group by Ellman method per gram of leaf (A) and per gram of extracts (B) as Cystein equivalent amount

Considering that the antioxidant activity of the soxlet-methanol extract is higher than the other methods, it may be associated with the high free thiol groups in this extract.

4. Conclusion

Olive leaf extract amounts and antioxidant activities are affected by the method and solvent used. TFC value, ABTS radical scavenging activity and total amount of sulfhydryl groups were found to be higher than the extracts obtained by other methods, especially in the extracts obtained by Soxlet-methanol extraction. According to the FRAP method, the extract prepared with 60% ethanol showed the highest reducing power based antioxidant activity. It has been revealed that deionized water is not suitable for obtaining an extract with high antioxidant activity.

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Authors' contributions: Seda Ağçam contributed to the experimental studies, the evaluation of the data and the preparation of the article. Gül Özyılmaz contributed to the organization of the study, the planning of the experimental studies, the evaluation of the data and the writing of the article.

Conflict of interest disclosure: There is no financial, commercial, legal or professional conflict of interest that could affect our research.

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