

Prediction of Quality Properties of Carob Fruit with FT-IR Spectroscopy

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Abstract

In this study it was aimed to determine the quality properties such as the total phenolic content, antioxidant activity and antimicrobial activity of the carob fruit and to predict some of these quality parameters using the Fourier-transform infrared (FT-IR) spectroscopy. For this purpose, extracts were prepared from carob samples with water, ethanol, methanol and 50-50 v/v mixture of methanol and ethanol with water. The total phenolic content was determined to be the highest in the methanol extract and the second highest in the carob water extract. The lowest antioxidant activity was observed in the samples extracted with ethanol and methanol solvents, respectively. The highest antioxidant activity was observed in the samples extracted with water, followed by 50-50 v/v ethanol and methanol extracts, respectively. In addition, the antimicrobial activity of carob samples extracted with different solvents on *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* microorganisms was also investigated in this study. While the ethanol:water (50%) extract (EW) showed an antimicrobial effect on the *Escherichia coli*, no antimicrobial effect was observed on other bacteria. Similarly, methanol (50%) extract (MW) showed antimicrobial effect only on *Pseudomonas aeruginosa*. Partial least squares (PLS) regression analysis of Fourier-transform infrared (FT-IR) spectroscopy data to predict total phenolic content and antioxidant activity revealed the good prediction ability of the model with R² values higher than 0.97.

Keywords: Carob, FT-IR spectroscopy, Partial least squares, Quality

1. INTRODUCTION

Carob, also known as the rich fruit of infertile land belongs to the Leguminosae family and grows usually in many mild and dry regions of the world (Gübbük et al., 2016; Papagiannopoulos et al., 2004). Its economically important parts are its seeds, fruit and tree. The production of carob fruit in the world is estimated at about 315.000 tonnes per year, which is distributed among Spain, Italy, Portugal, Morocco, Greece, Cyprus and Turkey (Santos et al., 2015). The use of fruit and seeds in industry increase its economic value. It has gained attention since it is a cheap source of various products such as bioethanol and citric acid and it is widely used as carob bean gum and locust bean gum in food industry. It has been determined that three different types of carob are grown in our country, namely "Etli", "Sisam" ve "Yabani" (Pekmezci et al., 2008). Although the carob fruit contains high

levels of sugar and protein, it contains low levels of fat. Carob pulp has a high content in total sugar, consisting of mainly sucrose, glucose, fructose and maltose. It contains vitamins A, B and various minerals (Hajaji et al., 2010). It is also used as a nutrients and feed for animals. Since carob is rich in soluble fiber, it can also increase the cholesterol profile (Vekiari et al., 2011).

Depending on development of technology, traditional methods used in scientific research have been replaced by modern methods. FT-IR spectroscopy is used as a rapid and specific detection method in many areas such as purity control, qualitative and quantitative analysis of organic substances, and structural analysis of macromolecules in many fields such as food, agriculture, medicine, and chemistry (Büyüksit and Kuleaşan, 2014). FT-IR spectroscopy is

advantageous in that it is a fast, simple and sensitive method without destruction of the sample structure (Davis and Mauer, 2010). With FT-IR, vibration movements such as bending and stretching between bonds are provided by the absorption of infrared light by cell compounds, and by comparing them with spectral databases, the unique spectra of the fingerprint-like functional group are obtained (Wenning et al., 2014). FT-IR and multivariate statistical analysis methods can be used together in the classification and quality control of foods. In the studies, it was used in analyzes in which adulteration was detected in foods (Vardin et al., 2008; Jaiswal et al., 2015), for food quality control analyzes, sugar analysis, (Anjos et al., 2015), determining the organic acid and sugar content of fruits, olive oil analyzes (Gürdeniz and Özen, 2009; Mata et al., 2012; Hirri et al., 2016) and determination of the polyphenol content of fruits (Park et al., 2015).

The aim of this study was to determine the quality properties such as the total phenolic content, antioxidant activity and antimicrobial activity of the carob fruits extracted with different solvents and to predict some of these quality parameters using the FT-IR spectroscopy.

2. MATERIAL AND METHOD

2.1. Material

In this study, carob (*Ceratonia siliqua* L.) fruits grown in Mersin province were obtained from a local market and used for analysis.

2.2. Preparation of extracts

Water, ethanol, methanol, 50% ethanol, 50% methanol were used as solvents for carob fruit extractions. Extracted carob samples were thoroughly ground without removing the seeds. For water extraction, 5 g of ground sample was weighed and left for extraction in 250 mL of boiled water for 45 minutes. For methanol, ethanol, methanol-water 50% (v/v), ethanol-water 50% (v/v) extraction, 1.5 g of ground sample was weighed and mixed in 15 mL solvent at room temperature for 45 minutes. The obtained extracts

were filtered using Whatman no:4 filter paper. The solvents were evaporated in a rotary evaporator (Büchi R-210) under vacuum at 80°C, and the extracts were dissolved in water at a final concentration of 50 mg/mL and stored at 4°C. The extraction process was carried out in two parallels.

2.3. Total phenolic content

The total phenolic content of carob extracts were measured according to the Folin –Ciocalteu spectrophotometric method (Singleton and Rossi, 1995). 0.2 mL of extract and 10 mL of 10% Folin-Ciocalteu solution were mixed. Then, 1.8 mL of distilled water was added and the solutions was left for 1 hour in the dark, then the absorbance was measured with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, California, USA) at 720 nm. The total phenolic content was calculated as µg gallic acid equivalent (µg GA/g).

2.4. Determination of antioxidant activity

Antioxidant activity was measured using DPPH (2,2-Diphenyl-1-picrylhydrazyl). Assays were carried out with respect to the methodology mentioned in an earlier study (Brand-Williams et al., 1995). In order to obtain a standard curve, a Trolox standard solution was used at different concentrations (10-100 µmol /L). The absorbance of the solution was measured at 515 by a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

2.5. Determination of antimicrobial activity

In this study, *Staphylococcus aureus* (*S. aureus*) ATCC 29213, *Escherichia coli* (*E.coli*) ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used as test organisms. These microorganisms were spread plated on tryptic soy agar and incubated at 35°C for 12–18 hours. After incubation, the turbidity of the bacterial suspension was adjusted to 108 CFU/mL (0.5 McFarland unit) with a sterile saline solution. The antimicrobial activities of carob fruit samples on the selected test organisms were determined with a standard disc-diffusion technique. Muller Hinton agar plates were prepared by inoculating bacterium into the

sterilized and cooled agar (45– 50°C). Twenty microliters of carob extract were added to sterile paper discs with a diameter of 6 mm. Discs were placed on inoculated agar plates and incubated at 35 °C for 18 –24 hours. Each experiment was carried out in duplicate. After incubation, inhibition zones were measured three times and the average was calculated (Öztürk et al., 2015).

2.6. FT-IR spectroscopy analysis method

For FT-IR analysis, samples were placed on ATR accessory of FT-IR spectroscopy device. Absorption spectra of FT-IR were obtained between 4,000 and 450 cm⁻¹, at resolution of spectral 4 cm⁻¹, as a result of 4 scan using FT-IR spectroscopy (Perkin-Elmer Spektrum two). Measurements were conducted twice for each sample.

2.7. Statistical analysis

Analysis of variance (ANOVA) was applied with Fisher's least significant difference method ($p < 0.05$ level) by using Minitab 17 software in order to evaluate total phenolic content and antioxidant activity of samples. PLS regression analyzes were performed using SIMCA 10.5 software (Umetrics, Sweden).

3. RESULTS AND DISCUSSION

3.1. Total phenolic content

The result of the total phenolic content of carob extracts was given in the Table 1. The highest amount of total phenolic content was determined as methanol extract (1.56 µg GAE/g), followed by water extract (1.44 µg GAE/g) (Figure 1). When the results were evaluated statistically, it was determined that there was a significant difference ($p < 0.05$) between the total phenolic content of the samples. In a study investigating the potential of carob extracts as lipid inhibitors in model food systems, the phenolic content of carob fruit extracts was found to range from 7.1 ± 0.9 mg GAE/100g to 382.0 ± 23.8 mg GAE/100 g. The most powerful solvents to recover phenolics from carob fruits were determined as water, acetone/water (50/50% v/v), acidic acetone, methanol and

ethanol/water (50/50% v/v) (Goulas and Georgiou, 2020). In our study, the total phenolic content of the extract prepared with aqueous methanol solvent was found to be higher than the ethanol and water extract. In another study, the antioxidant properties and total phenolic content of three types of carob tree leaves were investigated. Diethyl ether, dichloromethane, and ethyl acetate were used for extraction of three varieties of *Ceratonia siliqua* L. leaves. The total phenolic content of the samples was found to be between 0.75-2.64 g/L GAE. The highest total phenolic content was determined in ethyl acetate extract, and the lowest was observed in dichloromethane extracts in all samples (El-Hajaji et al., 2010). Ydjedd et al. (2017) investigated the phenolic compounds of *Ceratonia siliqua* L. at unripe and ripe stages using different solvents (hexane, chloroform, ethyl acetate, and acetone/water (70:30, v/v)). And they reported that the 70% aqueous acetone and the ethyl acetate extracts contained the highest phenolic content at unripe and ripe stages. In a study examining the antioxidant activity of carob seeds and the chemical composition of bean gum by-products, the aqueous acetic (70%) extract was determined to have the highest contents of total polyphenols (12.24 ± 0.02 mg/g dw) whereas the aqueous ethanolic (80%) extract had low total phenolic content (7.1 ± 0.01 mg/g of dw) (Mekhoukhe et al., 2018). Durazzo et al. (2014) indicated that the total polyphenol content of the aqueous organic extract of carob seeds were within the range of 71.03- 242.84 mg/ 100 g dw. In another study examining six carob seed varieties from Portugal, the total amount of phenolic substance was found to be between 19.8-28.7 mg/g (Custodio et al., 2011). Brahim et al. (2017) investigated total phenolic content of carob pods (pulp and seeds) and leaves of different ages. They noted that phenolic content of the tegument (26.3 mg GAE/g DM) was high in comparison with endosperm and germ (0.80 mg GAE/g DM and 1.33 mg GAE/g DM, respectively).

Mature leaf extract was also found to be important in terms of total polyphenols (45.26 mg GAE/g DM). The total phenolic content of the carob fruit determined in our study was found to be lower compared with above-mentioned previous studies. Table 1. Total phenolic content of carob samples extracted with different solvents.

Solvents	Total phenolic content ($\mu\text{g GA/g}$)
Water	19731,08 $\pm$ 0,86 ^a
Ethanol	2171,51 $\pm$ 0,20 ^d
Methanol	7323,08 $\pm$ 0,37 ^a
50 % Ethanol	19785,06 $\pm$ 1,20 ^b
50% Methanol	17076,37 $\pm$ 1,86 ^c

^{a-d}Different exponential letters on the same raw indicate a significant difference between the samples. ($p < 0.05$)

The differences of phenolic contents in carob could be attributed to the biological actors and environmental conditions carob (Khled khoudja, 2014).

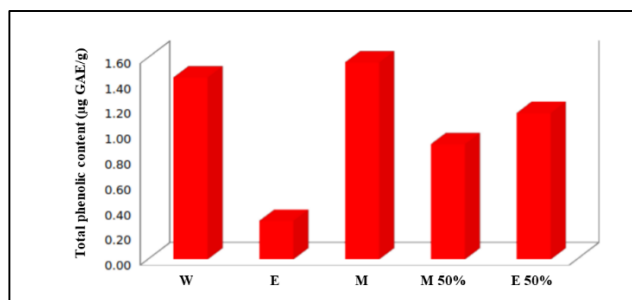


Figure 1. Total phenolic content of carob samples extracted with different solvents; W: Water, E: Ethanol, M: Methanol, M 50%: Methanol 50%, E 50%: Ethanol 50%.

3.2. Determination of antioxidant activity

According to antioxidant activity analysis, it can be seen that antioxidant activity of carob fruits reached the lowest values in pure ethanol and methanol solvent extracts, whereas extraction with water gave the best result in terms of antioxidant capacity (Figure 2), followed by the extraction with 50% ethanol and 50% methanol. When the results were evaluated statistically, it was determined that there was a significant difference ($p < 0.05$) between the antioxidant activity of the samples (Table 2).

In a study evaluating the extraction efficiency of fourteen solvents on the phenolic and flavonoid contents of carob fruit as well as on the antioxidant activity, The DPPH assay revealed that the most effective solvents for the recovery of carob antioxidants capacity were acetone–water (80:20, v/v), acidic acetone, acidic methanol, and water (Goulas & Georgiou, 2020). In another study, the antioxidant properties and total phenolic content of three types of carob tree leaves were investigated and DPPH radical scavenging activities of plant extracts was found to vary from 1.17 to 61.17 %. The highest antioxidant activity was observed in the ethyl acetate extract of the three categories of carob tree leaves (El-Hajaji et al., 2010). Ydjedd et al. (2017) compared *Ceratonia siliqua* L. pods, extracted using different solvents (hexane, chloroform, ethyl acetate, and acetone/water (70:30, v/v) at unripe and ripe stages. Among all extracts, the acetone 70% and ethyl acetate extracts for the ripening stage were found to have stronger antioxidant activity than other solvents. The DPPH radical scavenging activity of the acetone 70% extract was determined as $536.80 \pm 9.03 \mu\text{g/mL}$ and $795.32 \pm 3.95 \mu\text{g/mL}$ followed by ethyl-acetate extract with values of $792.54 \pm 5.67 \mu\text{g/mL}$ and $992.04 \pm 14.44 \mu\text{g/mL}$ for unripe and ripe stages, respectively. In a study where raw and processed legumes (dry heating, autoclaving, soaking and autoclaving) were extracted using different solvents, antioxidant activity was higher in raw legumes.

Table 2. Antioxidant activity of carob fruits samples.

Solvents	Antioxidant activity ($\mu\text{mol Trolox/g}$)
Water	19731,08 $\pm$ 0,86 ^a
Ethanol	2171,51 $\pm$ 0,20 ^d
Methanol	7323,08 $\pm$ 0,37 ^c
50 % Ethanol	19785,06 $\pm$ 1,20 ^a
50% Methanol	17076,37 $\pm$ 1,86 ^b

^{a-d}Different exponential letters on the same raw indicate a significant difference between the samples. ($p < 0.05$)

The antioxidant activity of processed legumes extracted with methanol was found higher than

acetone extract (Nithiyanantham et al., 2012). In our study, the antioxidant activity of the carob pod extract with water was found to be higher than other solvent extracts. Ben Othmen et al. (2019) studied the changes in the physicochemical properties of different Tunisian provenances of carob pods harvested at three ripening stages. Antioxidant activity of carob pods exhibited the highest levels which ranged from 32.78 ± 0.78 g TE/100 g dry weight (dw) to 20.98 ± 0.15 g TE/100 g dw at early ripening stage in this study. In a study evaluating bioactivities of different carob by-products to identify potential health benefits from their consumption, carob pod and seed peel was indicated to have higher antioxidant capacities compared to germ. The antioxidant activity of pod, germ and seed peel were determined as 257.85 ± 2.63 $\mu\text{mol TEAC/g DW}$, 142.0 ± 4.0 $\mu\text{mol TEAC/g DW}$ and 243.25 ± 8.74 $\mu\text{mol TEAC/g DW}$, respectively (Rico et al., 2019). Differences between DPPH activity of carob fruit extracts in our study and above-mentioned previous studies could be related with using different solvents in

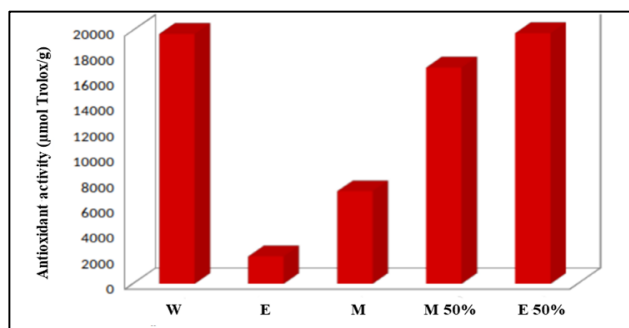


Figure 2. Antioxidant activity of carob fruit samples extracted with different solvents; W: Water, E: Ethanol, M: Methanol, M 50%: Methanol 50%, E 50%: Ethanol 50%.

extraction, ripening stage of carob fruit and also the phenolic contents and the harvest time. (Ydjedd et al., 2017).

3.3. Determination of antimicrobial activity

In our study, the antimicrobial effect of carob samples extracted using different solvents was investigated against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. Extraction with water and 50% ethanol extracts

have been found to have antimicrobial effects against *E. coli*, while the effect of other solvents could not be determined. And also, 50% methanol extract had antimicrobial effect against *P. aeruginosa*, whereas the effect of other solvents was not determined in extracts. Antimicrobial activity could not be observed when methanol concentration was increased from 15 mL to 25 mL and 50 mL. Moreover, carob fruits extracted with water, ethanol, methanol and their aqueous solutions did not show antimicrobial activity against *S. aureus*. While the aqueous extracts of carob samples had a high antimicrobial effect against *E. coli*, this effect was not observed when solvent concentration was reduced half and half. Whereas antimicrobial activity was not determined against *E. coli* for 100% ethanol extract, 9.44 mm zone diameter was measured in 50% ethanol extract. In a study in which flour and syrups obtained from carob (*Ceratonia siliqua* L.) and honey acacia (*Gleditsia triacanthos*) were characterized, the carob extract was shown to possess the highest antibacterial activity with inhibition zones of 22 mm against *L. monocytogenes*, 12 mm against *E. coli*, 10 mm against *S. enterica* and 13 mm against *S. aureus* (Fidan et al., 2019). Acetone and ethanol extracts of carob (*Ceratonia siliqua* L.) leaf and pods were evaluated for their inhibitory ability against the Gram negative *Pectobacterium atrosepticum* bacteria by Meziani et al. (2015). The acetone extract of carob leaf indicated the highest antimicrobial effect on *Pectobacterium atrosepticum*. The antimicrobial action of the 80% ethanol solution extract of carob pods was evaluated for urinary tract infection by Fadhil Abdul-Husin (2018) and the usage of ethanol carob pods extract exhibited the highest inhibition (diameter of the inhibition region > 25 mm) towards *E. coli*. In this study, the antimicrobial activity of 50% ethanol extracts have been found to have antimicrobial effects against *E. coli*, but was not determined with 100% ethanol. In a study in which the effect of three different concentration (15%, 20%, 25%) of watery and methanolic extract of

carob on the diarrheagenic *E. coli* isolated from diarrheal cases were analyzed, antibacterial activity of methanolic carob extract was higher than watery extract. Inhibition zone of each alcoholic and aqueous at 15% concentration was found as 5. 8 mm and 5. 6 mm respectively. When extract concentration was increased to 25%, in the inhibition zone of each alcoholic and aqueous extract increment was observed as 6. 9 mm and 6. 8 mm, respectively (Abdulkareem et al., 2020). In our study, the antimicrobial effect of methanol extract could not be determined against *E.coli*, but 9.44 mm inhibition zone diameter was measured in 50% ethanol extract. Abd Razik et al. (2012) found that the methanolic extract of *Ceratonia Siliqua* showed good antibacterial action against gram negative bacteria including *E. coli*.

3.4. FT-IR spectroscopy analysis

The use of FT-IR method as a fast and reliable method in estimating quality parameters such as total phenolic content and antioxidant activity of carob fruit extracted using different solvents by combining statistical analysis was investigated in this study. In addition, the macromolecular differences between the samples were revealed by examining the FT-IR spectrum. In the FT-IR spectrum, water, ethanol, ethanol/water, methanol and methanol/water carob extracts were evaluated in the range between 4000 and 450 cm^{-1} wavelength. FT-IR spectrum is given in Figure 3 and the functional groups corresponding to their spectra are given in Table 3.

Table 3. Functional groups corresponding to the spectra of the carob fruit (Doğan et al., 2007; Rohman and Che Man, 2010).

Wavelength (cm^{-1})	Functional group	Organic compounds
3386-3326	O-H and N-H	Polysaccharides, protein
1653	C=O	Protein
15550-950	C-C, C-O	Carbohydrates and ethanol

All samples gave similar spectral fingerprints for different solvent types. The major peaks were found at 3332, 1653 and 1087 cm^{-1} . The broad peak

between 3550-3200 cm^{-1} are associated with O-H stretching vibrations and N-H stretching (Amid A) vibrations of proteins. The peak at 1653 cm^{-1} is characterized due to the amide I vibrations of the structural proteins.

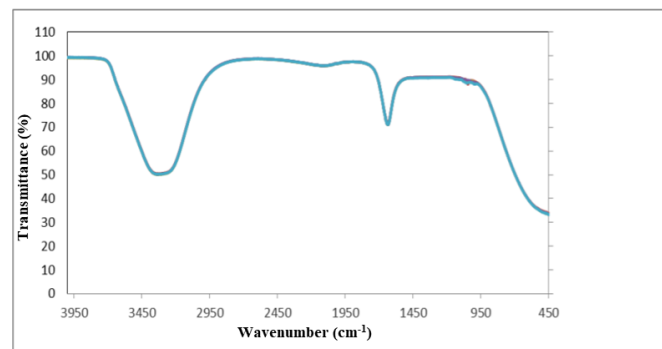


Figure 3. Typical mid-infrared spectra of carob fruit samples

The amide I domain is mainly associated with protein amide C=O stretching vibrations. This vibrational position is sensitive to protein conformation (Doğan et al., 2007). The fingerprint area between 1550– 950 cm^{-1} could be used to support the presence of C– C, C– O, and hydroxyl groups in carbohydrates and ethanol (Polshin et al., 2011; Wold et al., 2001). Partial least squares regression (PLS) analyses were carried out for the prediction of antioxidant activity and total phenolic content of the carob fruit extracts using FT-IR spectral data. A PLS regression plot of actual versus predicted analysis of antioxidant activity using FT-IR spectra is shown in Figure 4-a. The predictive ability of the model R^2 value was found to be 0.9893, revealing that it was a good model. And also PLS regression plot of actual versus predicted total phenolic content of carob fruit analyses using FT-IR spectra was shown in Figure 4-b. The predictive ability of the model represented by R^2 value was found to be 0.9982. In a study in which effect of solvent type on extraction of walnut green shells bioactive compounds were predicted by FT-IR spectroscopy, the results of PLS regression analysis revealed that total phenol content and antioxidant activity of extracts were predicted properly with R^2 value of 0,993 for calibration. The results indicated that PLS models have high predictive capability as fast and reliable method

(Kadiroğlu and Ekici, 2018). In a study in which the origin of carob was classified by FT-IR spectroscopy and chemometric methods,

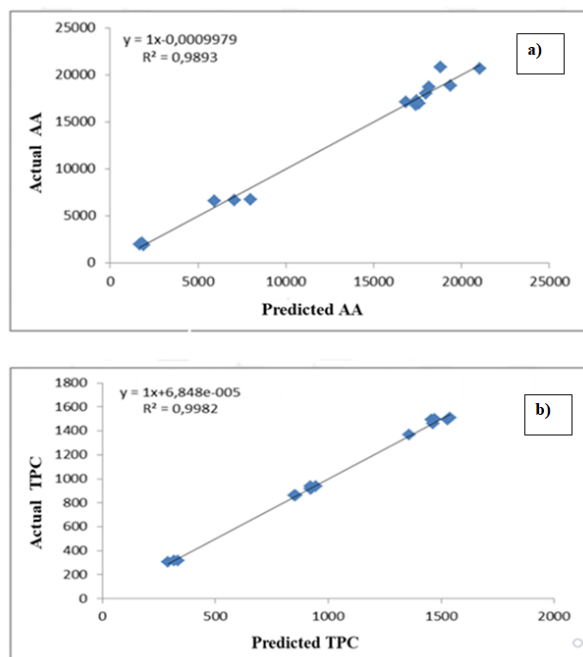


Figure 4. PLS regression plot of actual versus predicted: a) AA analyses ;b) TPC analyses using FT-IR spectra. AA: antioxidant activity; TPC: Total phenolic compounds

carob fruits obtained from 7 different countries were analyzed. The seeds and fleshy parts of the samples taken from Cyprus, Greece, Italy, Spain, Turkey, Jordan and Palestine were examined. In general, the most reliable classification could be made with the PLS regression model. With chemometric methods, it was possible to distinguish samples according to the origin and species of the carob fruit (Christou et al., 2017). In another study in which the origin of carob was analyzed by FT-IR spectroscopy and chemometric methods, the seeds and fleshy parts of the fruits obtained from various carob trees in the Morocco region were analyzed. Samples were taken under the same conditions and in the same amount, and it was determined that the combined use of spectroscopic and chemometric methods were sufficient to determine the origin and subclass of carob (Alabdi et al., 2011). In a study investigating the detection of adulteration of carob, mulberry and grape molasses, FT-IR spectroscopy method and multivariate methods were combined. FT-IR

analysis of original and glucose syrup mixed samples were performed separately and spectral data were analyzed by PLS method. Samples were distinguished at wavelengths of 600–4000 cm^{-1} . R^2 values of calibration was found to be 0.988 for grape molasses, 0.998 for carob molasses and 0.996 for mulberry molasses (Yaman & Veliöğlu, 2019).

4. CONCLUSION

In this study, antioxidant activity, total phenolic content and antimicrobial activities of carob fruit which was extracted using different solvents were investigated and the antioxidant activity and total phenolic content of the extracts were predicted by analyzing the FT-IR spectroscopy data with PLS regression method. The highest amount of total phenolic content was determined in the methanol extract, followed by the water extract. The lowest and highest antioxidant activity was observed in carob extracted with ethanol and water, respectively. When the antimicrobial effects of carob samples against *S. aureus*, *P. aeruginosa* and *E. coli* microorganisms were examined; water and 50% ethanol extracts had an antimicrobial activity against *E. coli* while the antimicrobial effect against *P. aeruginosa* bacteria was found in 50% methanol extract. Moreover, any of the water, ethanol, methanol and their aqueous solutions had no antimicrobial effect on *S. aureus*. All samples gave similar spectral fingerprints for different solvent types in FT-IR spectroscopic analysis. A PLS regression plot of actual versus predicted antioxidant activity and total phenolic content analyses using FT-IR spectra and R^2 value was found to be 0.9893 for antioxidant and 0.9982 for total phenolic content, revealing that it was a good model. The results of PLS regression analyzes showed that the FT-IR method could be a rapid and reliable screening method to predict antioxidant capacity, total phenolic content of carob extracts.

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