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Contact (Editor in Chief)
Yücel KADIOĞLU
Atatürk University, Faculty of Pharmacy, Department of Analytical Chemistry, Erzurum, Türkiye
✉ yucel@atauni.edu.tr
https://dergipark.org.tr/tr/pub/pharmata

Contact (Publisher)
Atatürk University
Atatürk University, Erzurum, Türkiye
Atatürk Üniversitesi Rektörlüğü 25240 Erzurum, Türkiye
✉ ataunijournals@atauni.edu.tr
https://bilimseldergiler.atauni.edu.tr
☎ +90 442 231 15 16

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The scope of the journal encompasses various topics, including but not limited to:

1. Pharmacognosy
2. Pharmaceutical botany
3. Pharmaceutical technology
4. Pharmacology
5. Pharmaceutical toxicology
6. Analytical chemistry
7. Biochemistry and medical biochemistry
8. Medicinal chemistry
9. Other areas: Health sciences (basic and clinical medical sciences, nursing, pharmaceutical care, etc.), clinical pharmacy, pharmacy management, biotechnology products, engineered cells, food and drug chemistry.

The target audience of the journal includes researchers and specialists who have an interest in or are working in any of the fields covered by the journal's scope.

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Editor in Chief: Yücel KADIOĞLU
Address: Department of Analytical Chemistry, Atatürk University, Faculty of Pharmacy, Erzurum, Türkiye
E-mail: yucel@atauni.edu.tr

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Address: Atatürk University, Yakutiye, Erzurum, Türkiye
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Vitamin C, Sugar Content, Color Intensity and Some Physicochemical Properties of Watermelon and Orange Peels

Memnune ŞENGÜL[1]
Selvican GÖKÇE[2]
İsa Arslan KARAKÜTÜK[3]

[1]Department of Food Engineering, Faculty of Agriculture, Atatürk University, Erzurum, Türkiye
[2]Department of Food Engineering, Faculty of Agriculture, Atatürk University, Erzurum, Türkiye;
Erzurum Food Control and Laboratory Directorate, Erzurum, Türkiye
[3]Department of Food Engineering, Faculty of Agriculture, Atatürk University, Erzurum, Türkiye

ABSTRACT

Objective: The peels produced as waste during the consumption and processing of fruits and vegetables cause both nutrient losses and environmental pollution. Due to their high nutritional content, raw material potential, and the aim of reducing environmental pollution, the use of certain fruit and vegetable peels in various forms and applications in the food industry is becoming increasingly widespread. This study investigates the usability of watermelon and orange peels in terms of their functionality by analyzing their vitamin C and sugar contents, color intensities, and some physicochemical properties.

Methods: Glucose, fructose, and sucrose contents were determined by High-Performance Liquid Chromatography (HPLC), vitamin C content by titrimetric method, protein content by the Kjeldahl method, color intensities by a colorimeter, and other properties using standard methods.

Results: In watermelon peel, the dry matter content was found to be 3.57±0.27%, total acidity 0.59±0.01%, pH 5.74±0.02, total ash 0.65±0.02%, protein 1.76±0.01%, glucose 0.54±0.02%, fructose 1.26±0.06%, L* value 72.45±1.10, a* value -14.81±1.30, and b* value 35.91±3.46. Vitamin C and sucrose were not detected in watermelon peel. In orange peel, the total dry matter (TDM) was 23.31±0.08%, total acidity 1.22±0.02%, pH 5.09±0.02, total ash 1.12±0.05%, vitamin C 122.33±2.52 mg/100g, protein 2.41±0.08%, glucose 2.18±0.12%, fructose 1.89±0.03%, and sucrose 0.28%. The color values were L* 68.92±0.47, a* 19.23±4.09, and b* 63.85±1.8.

Conclusion: Based on the findings of this study, orange peel, which is produced in large quantities as waste in the industry, contains more nutrients, exhibits better color intensity, and has a significantly higher vitamin C content compared to watermelon peel. Therefore, orange peel can be used as a natural antioxidant and color source in the food, pharmaceutical, and cosmetic industries.

Keywords: Orange Peel, Physicochemical Properties, Sugar, Vitamin C, Watermelon Peel

INTRODUCTION

As the global population increases, food consumption rises proportionally, leading to a parallel increase in agricultural waste. This situation contributes to the depletion of limited natural resources and environmental pollution.[1] Food waste, while causing economic and environmental problems such as greenhouse gas emissions and inefficient use of water and soil, also represents valuable resources rich in food components and various bioactive compounds, including phenolic and antioxidant compounds.[2] Transforming these resources into value-added products through various technologies across different industries is crucial for preventing environmental pollution, ensuring sustainability in food resources, and reducing food loss and waste.[3]

Food losses occur during production, processing, retail sales, and as a result of improper storage conditions or overproduction/purchasing in restaurants or households. Parts such as peels, seeds, skins, stems, and leaves of agricultural products generate significant waste potential worldwide.

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Corresponding author:
Selvican GÖKÇE
E-mail: selvican.pezik@gmail.com

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Similarly, losses occur during the preparation or processing of fruits and vegetables for consumption, contributing to environmental pollution through both household and industrial waste. However, fruits, vegetables, and their various parts (peels, stems, seeds) contain a wide range of nutrients, including vitamins, minerals, carbohydrates, dietary fiber, phenolic and antioxidant compounds, pectic substances, essential fatty acids, and beneficial pigments, making them important for human nutrition.

In Turkey and globally, significant amounts of watermelon (Citrullus lanatus) and citrus fruits are produced and consumed. However, a large portion of their peels is discarded without being utilized. Approximately 30% of a watermelon's total weight consists of its peel.[4] Watermelon rind is rich in bioactive components such as dietary fiber, various minerals, vitamins, phytochemicals, and phenolic compounds. Some studies have reported that compounds found in watermelon rind help protect the body against certain types of cancer. The phenolic compounds in watermelon rind are noted for lowering low-density lipoprotein (LDL) levels in the blood, while pectin cleanses intestinal walls and facilitates the synthesis of short-chain fatty acids in the intestines.[5]

Orange peel, another significant waste product, is rich in bioactive compounds and pectic substances, making it a promising material for the development of functional foods. The peel accounts for approximately 15% of an orange's weight.[6]. When only the juice is extracted, the pulp and peel result in a significant amount of waste. Orange peel contains essential minerals like iron, calcium, magnesium, potassium, and copper, as well as important vitamins such as vitamin C.[1] It has been reported to contain; 8.120±0.120 g/100g protein, 46.241±0.015 g/100g total carbohydrates in its dry matter. Research indicates that orange peel contains antioxidant such as flavonoids (2.685±0.062 g/100 g d.b) and vitamin C (0.105±0.003 g/100 g d.b).[7]

Rich in pectin, cellulose, hemicellulose, soluble sugars, carotenoids, flavonoids, and essential oils, orange peel is a valuable by-product with potential uses in the food, biotechnology, and energy industries.[8] Its antioxidant properties reduce the risk of cancers caused by oxidative stress, and its dietary fiber positively affects the digestive system. Additionally, the vitamin C and potassium content in orange peel contribute to skin health. In recent years, studies on utilizing orange peel in various forms and shapes within the food industry have become increasingly common.[9-11]

Vitamin C, also known as ascorbic acid, is a water-soluble essential vitamin that cannot be synthesized by the human body and is naturally found in various vegetables and fruits.[12,13] Due to its high vitamin C content and high per capita consumption, citrus fruits are undoubtedly the most important source of vitamin C in human nutrition. Therefore, citrus fruits are a significant source of vitamin C in the human diet. As a primary antioxidant compound, the vitamin C concentration is one of the indicators of the nutritional quality of citrus fruits and their derivative products.[14-16] Interestingly, vitamin C accounts for more than 65% of the antioxidant and free radical activity in many fruits and their beverages.[17,18] On the other hand, it is known that vitamin C directly scavenges free radicals such as hydroxyl radicals, superoxide, singlet oxygen, and hydrogen peroxide, thereby reducing the damage caused by these radicals.[19] One study noted that in most biological systems, vitamin C protects compounds both in intracellular and extracellular areas.[20] Additionally, vitamin C lowers plasma cholesterol levels and increases the absorption of inorganic iron.[21,22]

This study aims to determine the vitamin C content, glucose, fructose, and sucrose levels using HPLC as well as the color intensity and some physicochemical properties of watermelon and orange peels, which are considered food waste.

METHODS

Materials

In this study, watermelon and orange fruits were procured from the local markets in Erzurum province. Watermelons (Citrullus lanatus) were washed with tap water, dried with paper towels, and peeled to separate the rind from the flesh (entirely red part). The white rind and green rind were separated using a knife. The white rinds were blended into smaller pieces and used in analyses and extractions. Valencia oranges were used as the orange variety in the study. Both the flavedo and albedo layers of the orange peels were utilized. Oranges were washed with tap water, dried with paper towels, and peeled. The flavedo and albedo parts of the peels were blended together and used in analyses and extractions.

Physicochemical Analyses

The physicochemical properties of watermelon and orange peels, including total dry matter (TDM), total ash (total mineral content), titratable acidity (expressed as citric acid, g citric acid/100 mL), vitamin C content, and pH values, were determined according to Cemeroğlu (2013).[23]

The total nitrogen content of the peels was measured using the Kjeldahl method, and the protein content was calculated by multipliying the result by a factor of 6.25.[24]

The color intensities of the watermelon and orange peels were determined using a three-dimensional Konica Minolta Colorimeter (Konica Minolta CR400, Korea). In the color scale representing values L*, a*, b*, H^0 and C*:

- L* = 100 represents white, L* = 0 represents black
- +a* indicates red, -a* indicates green.
- +b* represents yellow, -b* represents blue
- Hue angle (H0) indicates color tone
- Chroma (C*) represents saturation. The blended peels were placed on a white surface for color measurements.

Fructose, Glucose, and Sucrose Determination

Two grams of blended peel were weighed and transferred into 50 mL volumetric flasks. A small amount of ultrapure water was added, and the mixture was stirred before the volume was brought up to 50 mL with ultrapure water. The mixture was shaken thoroughly and filtered through 0.45 µm filters into vials, which were then analyzed using an HPLC system (Shimadzu LC-2050C-3D model).[1]

HPLC Conditions:

- Column: C-18 (4.6 mm × 150 mm)
- Mobile Phase: Water/Acetonitrile (25/75 mixture)
- Detector: HPLC-RID
- Wavelength: 255 nm
- Injection Volume: 20 µL
- Flow Rate: 1 mL/min

Statistical Analyses

Statistical analyses were conducted using SPSS (IBM SPSS Statistics 25, 2020). The results were subjected to correlation and variance analyses. The significance of differences between groups was determined using Duncan's Multiple Comparison Test at a significance level of 0.05. Results are presented as mean values ± standard deviation.

RESULTS AND DISCUSSION

The total dry matter (TDM), total ash (mineral content), titratable acidity, protein, vitamin C content, and pH values of watermelon and orange peels are presented in Table 1. The TDM and total ash content of fruit peels were found to be statistically significantly different (P<.01), with orange peels having higher TDM and ash content than watermelon peels. Previous studies reported TDM content in watermelon rinds as 6.21–7.40% and 11.86% in orange peels.[25,26] The ash content of watermelon rinds has been reported as 7.9%, 10.2 g/100 g, and 13.2 g/100 g in various studies, whereas orange peels have shown ash content of 5.34% and 3.17 g/100 g dry weight.[7,26] Previous studies reported that from the spring season in plants total plant dry weight increased rapidly from they reported. Increasing temperature and light conditions in this increase has been effective. As a matter of fact, increasing temperature and light they stated that it affected plant dry weight.[27]

The pH values and titration acidity of watermelon rind and orange peel were found to be statistically significantly different (P<.01). The pH values of watermelon rind and orange peel were 5.74±0.02 and 5.09±0.02, respectively; and the titration acidity values were 0.59±0.01% and 1.22±0.02%, respectively. It was determined that the pH value of orange peel was lower than that of watermelon rind, while the titration acidity was higher (Table 1). Studies on orange peel reported a pH value of 5.67 and a titration acidity of 2.98 g/100g.[7,28] In another study, the titration acidity of watermelon rind was reported to be 0.360 g/100g.[29]

Fruits are generally not considered a source of protein.[30] It was determined that the protein content of fruit peels was statistically significantly different (P<.01), with orange peel containing a higher amount of protein (Table 1). In a study, the protein content of watermelon rind was found to be 0.15% in its fresh form, 2.067% in freeze-dried form, and 1.83% in hot air-dried form.[31] Different studies also reported the protein content of orange peel as 6.08%, 6.77 g/100g and 8.12 g/100g dry weight.[7,32] Factors such as the natural environment or greenhouse effect, pesticide use, fertilization, and whether the fruit is grown in season or not can cause variations in nutrient content.[33]

Ascorbic acid (vitamin C) is an important nutrient due to its vitamin activity and strong antioxidant properties. Antioxidants are substances that can prevent or partially delay oxidation. Thanks to these components, oxidation damage in the human body is minimized.[34]. Ascorbic acid is one of the components that prevent the formation of free radicals or inhibit the free radicals that are formed. For consumers, antioxidant capacity has become an element of quality.[35,36]

In the study, it was determined that orange peel contains 122.33±2.52 mg/100g of vitamin C, while no vitamin C was detected in watermelon rind (Table 1).

In a previous study, the ascorbic acid contents of orange, lemon, and grapefruit peels were examined, and the vitamin C content of orange peel was reported as 433.11±16.03 ppm.[37] The recommended daily intake (RDA) of ascorbic acid for healthy individuals to meet cellular requirements and reduce the risk of cardiovascular and neurodegenerative diseases, cancer, and stroke is 100–120 mg/day.[38] For example, it was found that one cup of orange juice (200 mL) provides 30% to 80% of the recommended daily amount of vitamin C.[39] Some previous research suggests that liver damage may be caused by free radical metabolites.[40] Vitamin C scavenges free radicals in a powerful way.[41] It is thought that vitamin C in orange peel may play an important role in scavenging free radicals. As seen in our study, 100g of orange peel can meet the daily vitamin C requirement of a healthy individual. Therefore, orange peels can be used as a good source of vitamin C by adding them to various formulations in the food, pharmaceutical, and cosmetic industries.

The sugars present in fruits and vegetables are almost entirely composed of glucose and fructose, along with small amounts of sucrose and the hexose mannose. The proportions of these sugars vary depending on the type and variety of the fruit or vegetable. Fruit peels generally have a lower sugar composition than the fleshy parts of the fruit.[23] The amounts of glucose, fructose, and sucrose in watermelon and orange peels are provided in Table 2. In the study, a statistically significant difference (P<.01) was found between the fructose, glucose, and sucrose content of watermelon and orange peels (Table 2). In one study, the amount of fructose in orange peel was reported as 1.72 g/L, and glucose was 6.26 g/L.[42] In another study, where watermelon rind juice was used, the fructose content ranged from 1.14-4.97 mg/mL, glucose content from 1.18-4.90 mg/mL, and sucrose content from 1.12-4.96 mg/mL.[43]

Color is an important quality parameter in food products. The color of fruits and vegetables can be influenced by chemical, biochemical, microbial, and physical changes that occur during growth, ripening, post-harvest storage, and processing.[44] In color analysis, L* represents lightness (0 = black/dark, 100 = white/light), +a* indicates red, -a* indicates green, +b* indicates yellow, and -b* indicates blue color intensities.[45] The C* value (chroma) indicates color intensity, and the Hº (Hue angle) specifies the color tone. Additionally, measured color values, including H0 (hue) and chroma represented by C*, were determined. Chroma is a dimensionless value that shows the saturation of color. In dull colors, the chroma value is lower, while in vivid colors, the chroma value is higher.[46]

The color intensities of watermelon and orange peels are shown in Table 3. A significant statistical difference (P<.01) was observed between the L*, a*, b*, C*, and H° values of the fruit peels. Orange peel was found to be darker (L* value) and more yellow (+b* value) compared to watermelon peel (Table 3). In one study, the L* value of orange peels was found to be 60.73±0.78, a* value -27.34±0.10, b* value 59.03±0.17, C* value 65.05a±0.19, and h value 65.04±0.12.51 The results of our study are similar to previously conducted studies. In another study, watermelon peels were subjected to quick freezing, and the resulting color values were reported as L* 77.22±0.2, a* -0.38±0.2, b* 19.8±0.2, C* 45.05±0.12, and H° 48.69±0.2.37 They reported that the quick freezing process led to an increase in the L* values, meaning a higher lightness of color.

Table 1. Physicochemical Properties and Vitamin C Content of Watermelon and Orange Peels

Fruit Peel	Total Dry Matter (%)	Total Ash (%)	pH	Titratable Acidity (%)	Protein (%)	Vitamin C (mg/100 g)
Watermelon Peel	3.57±0.27[b]	0.65±0.02[b]	5.74±0.02[a]	0.59±0.01[b]	1.76±0.01[b]	ND
Orange Peel	23.31±0.08[a]	1.12±0.05[a]	5.09±0.02[b]	1.22±0.02[a]	2.41±0.08[a]	122.33±2.52
Significance	**	**	**	**	**	**

**: Statistically highly significant (P<.01), ND: Not detected

[a, b]: Means in the same column with different letters are significantly different.

Table 2. Glucose, Fructose, and Sucrose Contents of Watermelon and Orange Peels

Fruit Peel	Glucose (%)	Fructose (%)	Sucrose (%)
Watermelon Peel	0.54±0.02[b]	1.26±0.06[b]	ND
Orange Peel	2.18±0.12[a]	1.89±0.03[a]	0.28
Significance	**	**	**

**: Statistically highly significant (P<.01), ND: Not detected

[a, b]: Means in the same column with different letters are significantly different.

Table 3. Color Intensity of Watermelon and Orange Peels

Fruit Peel	L* (Lightness)	a* (Red/Green)	b* (Yellow/Blue)	C* (Chroma)	H° (Hue)
Watermelon Peel	72.45±1.10[a]	-14.81±1.30[a]	+35.91±3.46[b]	38.84±3.70[b]	112.42±0.17[a]
Orange Peel	68.92±0.47[b]	-19.23±4.09[b]	+63.85±1.80[a]	66.75±2.40[a]	73.29±3.27[b]
Significance	**	**	**	**	**

**: Statistically highly significant (P<.01)

[a, b]: Means in the same column with different letters are significantly different

CONCLUSION

Natural and synthetic vitamin C and antioxidants are used in many products in the food, pharmaceutical, and cosmetic industries. In recent years, scientific research has focused on natural sources of antioxidants due to the potential harms of synthetic additives and antioxidants to human health. This study investigated the potential of watermelon and orange peels, which are significant industrial and processing by-products, as sources of natural vitamin C, antioxidants, and certain nutrients. The results of the study show that watermelon peel does not contain vitamin C and sucrose, while orange peel has a higher dry matter content than watermelon peel. Orange peel also contains more mineral substances, acidity, protein, glucose, and fructose, has better color intensity, and contains a high amount of vitamin C. Therefore, it can be concluded that orange peel can be used as a source of natural antioxidants and color in the food, pharmaceutical, and cosmetic industries.

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A Comparison of the Antioxidant Activities of Aqueous and Ethanol Extracts of Turkish Black Tea at Different Processing Stages

Meysera BAKIRCI[1]
Abdullah Al FAYSAL[1]
Ayşegül GÖLCÜ[1]

[1]Department of Chemistry, İstanbul Technical University, Faculty of Sciences and Letters, İstanbul, Türkiye

ABSTRACT

Objective: Türkiye is a country where many plants can grow due to its location and climate diversity. Rize, located in the Black Sea region, stands out notably in tea cultivation due to high rainfall and humidity. In recent years, studies in this field have acquired momentum because of interest in natural antioxidants. In this study, the antioxidant properties of the different processing stages of black tea grown in Rize were comparatively examined.

Methods: This study presents the best combinations of stirring time and solvent to obtain extracts from tea samples in the most efficient way. Five methods were used for the measurement of antioxidant activity, which are as follows: Cupric ion reducing antioxidant capacity (CUPRAC) method, Ferric reducing antioxidant potential (FRAP) method, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity method, Ferrous ion chelating (FIC) method, and total phenolic content (TPC) method.

Results: The results obtained from CUPRAC, DPPH, and TPC methods strongly support each other. The other two methods also gave similar results within themselves. Sample 6 exhibited the highest antioxidant activity with results of 2873.76±4.25 mM AAE/g (CUPRAC), 133.29±8.58 µg/mL (DPPH), and 74.39±1.69 mg GAE/g (TPC). In contrast, the green leaves demonstrated the lowest antioxidant activity, with results of 763.17±2.93 mM AAE/g (CUPRAC), 463.25±4.10 µg/mL (DPPH), and 14.10±3.70 mg GAE/g (TPC).

Conclusion: The experimental study points out that black tea consumption is beneficial because it helps the body to get natural antioxidants.

Keywords: Antioxidant, Antioxidant Activity, Black Tea, *Camellia Sinensis*, Plant Extract

INTRODUCTION

The tea plant known as *Camellia sinensis* is one of the most consumed beverages in the world for reasons such as being alcohol-free and affordable.[1] In addition to these reasons, it has often been the number one drink in societies, due to its advantageous features such as warming people up on cold winter days and creating an environment for socialization. According to the results of some studies, catechins, theaflavin, and strychnine, which are tea polyphenols, prevent influenza viral infectiousness and are also effective against colds.[2] On the basis of the tea formed on the tea plant, three types of tea are produced as a result of the different levels of oxidation carried out during processing: unoxidized tea, oxidized tea, and semi-oxidized tea; in simpler terms, green tea, black tea, and oolong tea.[3] According to 2016 data, Türkiye's per capita tea consumption was approximately six times that of China, peaking at 6.96 kilograms, and according to 2019 data, Türkiye again ranks first in per capita tea consumption. Therefore, it has been a part of Turkish culture for many years and has solidified its place as an indispensable habit in daily life.

Tea production is highest in the coastal area from the city of Rize to the Georgian border, but the surrounding cities of Trabzon, Artvin, and Giresun also contribute to the country's tea harvest, and therefore most of the people in these regions earn their living from the tea harvest.[4] The tea harvested in the city of Rize is processed and consumed as black tea. Although various teas such as chamomile tea, fennel tea, linden tea, and rosehip tea are available in markets, black tea is still the most popular and consumed tea.[5]

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Corresponding author:
Ayşegül GÖLCÜ
E-mail:
aysgolcu@itu.edu.tr

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After the tea is harvested and sent to the factory, it goes through withering, rolling, oxidation, drying, and sifting processes (Figure 1). A great number of components in the structure of the tea plant may change or differ in their amounts after these processes. It is stated that the main antioxidant active components of black tea are polyphenols, mainly catechins, flavins, rubigins, gallic acid, and flavonoids. The general observation throughout the processing of tea and making it available for marketing is that catechins are generally oxidized to create the flavins, the rubigins, and the brownins, and the polyphenol content diminishes. During the fermentation step to make the tea drinkable, several oxidation reactions occur and these reactions significantly change the antioxidant content in black tea.[6] An average Turkish person consumes 1250 cups of tea per year, which corresponds to approximately 4 cups of tea per day.[4] Drinking tea has been believed to be beneficial to health since ancient times and modern medical research confirms this belief due to the polyphenols in tea.[7] Moreover, it can be argued that the healing properties of black tea are not a complete myth, because research on diabetic rats has shown that black tea has powerful antioxidant and antidiabetic activity.[8]

numerous diseases in the body.[9] The function of antioxidants can fundamentally be summarized as avoiding the creation of reactive species and eliminating free radicals.[10] It is clear that antioxidants are necessary to protect against the harm of free radicals to human health.[11] With this awareness, conscious consumption of natural products such as fruits, vegetables, and tea has increased in recent years.[12] An example of the main causes of free radical formation in the body is respiration, including cellular respiration, because some of the oxygen spent during breathing contributes to radical formation.[13] Respiration is a critical action for the life of living organisms and cannot be stopped, therefore the formation of free radicals in the body remains a continuous action, which means that the struggle with free radicals will not end throughout life. The structure of free radicals, which are examined in three groups such as reactive oxygen species, reactive nitrogen species, and reactive sulfur species, consists of atoms, molecules, or ions with unpaired electrons and they are very willing to give chemical reactions with other molecules, therefore they are described as unstable and active.[9] As a result, radicals are active species that exist in the body and are formed frequently but are unwanted and harmful in their presence. In order to cope with these harmful reagents, antioxidants are needed, which we can obtain from various plants in nature.

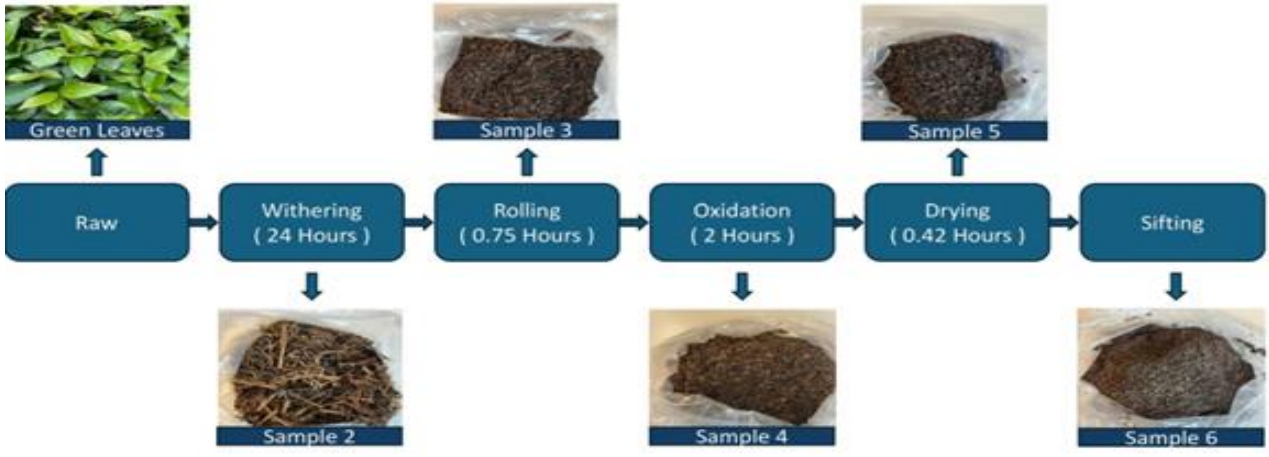

Figure 1. Sampling of the tea leaves during processing

Highly reactive radicals are known to have a significant impact on living organisms, contributing to the onset of various diseases within the human body. Living organisms require antioxidant species to cope with highly damaging radicals. When the balance that needs to be maintained between undesirable radicals and antioxidant species is achieved, the problem is eliminated. However, if this balance is not achieved and the radicals are more than the antioxidants, this situation results in the existence of

Tea is one of the most consumed natural products with antioxidant properties in daily life.[14] Nonetheless, as mentioned earlier, not every antioxidant substance found in the tea plant is found in the same density at every stage of the tea. It would not be correct to claim that all the antioxidant substances listed in the teas purchased and drunk from the market are abundant and very beneficial for health. Therefore, the issue that needs to be investigated is the antioxidant properties of the tea in its different processing stages.

In order to determine the antioxidant properties of black tea grown in Rize, black tea samples were taken from a tea factory in Rize before starting this experiment. Since there are five stages in tea processing, a sample was obtained at the end of each stage, and in addition to these, fresh green leaf samples were obtained, resulting in a total of six tea samples. After obtaining the samples, the most suitable solvent and appropriate mixing times were determined for each. The solution of each sample was prepared according to the determined data. Antioxidant properties were determined using a total of five methods. These methods are as follows: Cupric ion reducing antioxidant capacity (CUPRAC) method, Ferric reducing antioxidant potential (FRAP) method, DPPH radical scavenging activity method, Ferrous ion chelating (FIC) method, and lastly total phenolic content (TPC) method. Our objective was to assess the antioxidant activity of tea samples at various stages and determine the stage at which the antioxidant activity would be maximum.

METHODS

Materials and Preparation of Extracts

The experiment was initiated to compare the extraction efficiency of tea samples obtained from Rize and to determine the most appropriate solvent and mixing time. Initially, 0.1 gram of tea sample was weighed and 10 mL of distilled water was added. This mixture was stirred for 10 minutes with a magnetic stirrer. Subsequently, it was filtered using ordinary filter paper. 2 mL of this solution was transferred to pre-weighed watch glasses. Then, the watch glasses were placed in an oven set to 200°C and left in the oven until the samples were dried. When the solutions dried, the watch glasses were taken out of the oven and their weights were measured and noted again.

This procedure was repeated for 20 minutes, 30 minutes, 40 minutes, and 60 minutes mixing times. Similarly, since it is known that ethanol yields effective results when used for sample extraction in antioxidant activity determination, this procedure was repeated in a manner suitable for the properties of ethanol.[15]

Cupric Ion Reducing Antioxidant Capacity (CUPRAC) Method

The CUPRAC method is an effective method of expressing total antioxidant activity for many polyphenols and the chromogen used is the bis(neocuproin) copper (II) cation [$Cu(Nc)_2^{2+}$], which is reduced to the bis(neocuproin) copper (I) chelate [$Cu(Nc)_2^{2+}$], which can be read at 450 nm.[16]

Solutions of all tea samples were prepared and diluted to a concentration of 0.5 mg/mL. Then, a 1 mM concentration of ascorbic acid solution was prepared as a stock solution using distilled water. Afterwards, appropriate amounts were taken from this prepared stock solution to prepare a series at concentrations of 100, 200, 500, 800, and 1000 µM and diluted with water, and the final volume of all was adjusted to 1 mL. Subsequently, 10 mM Copper (II) chloride solution was prepared with water. An aqueous solution of ammonium acetate was prepared as a buffer solution with a pH of 7. Finally, neocuproine solution with 7.5 mM concentration was prepared using ethanol as solvent. After all solutions were prepared, 25 mL of each of copper chloride, neocuproine, and ammonium acetate buffer were taken and mixed in a beaker at a ratio of 1:1:1. Then, 0.5 mL of extract and standard solutions were transferred to falcon tubes, and 3 mL of the reaction mixture was added to these tubes and then all were vortexed. To prepare the blank solution, 1 mL of neocuproine solution, 1 ml of ammonium acetate buffer, 1 mL of water and 0.5 mL of sample solution were mixed in a falcon tube. The tubes were incubated at room temperature for 30 minutes. Finally, absorbance versus prepared blank was read at 450 nm and recorded. All procedures were carried out in triplicate, and the results were expressed as mM ascorbic acid equivalent per extract.

Ferric Reducing Antioxidant Potential (FRAP) Method

Another antioxidant determination method, the FRAP method, is based on the reduction of Fe^{3+}-tripyridyltriazine, a colorless ferric complex, to Fe^{2+}-tripyridyltriazine, a blue ferrous complex, in a low pH environment and by means of electron-donating antioxidants, and this reduction is observed by measuring the absorbance at 593 nm.[17] Initially, 40 mM HCl solution was prepared using water as the solvent. Then, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution was prepared using the freshly prepared 40 mM HCl solution. Subsequently, 20 mM $FeCl_3.6H_2O$ solution was prepared using distilled water as the solvent. Afterward, 0.3 M (pH 3.6) acetate buffer was prepared using water as solvent. After all the mentioned solutions were prepared, the FRAP agent was prepared by mixing the pH 3.6 buffer, 20 mM $FeCl_3.6H_2O$ solution, and 10 mM TPTZ solution with a ratio of 10:1:1 and incubated at 37 °C for 10 minutes. 1 mM $FeSO_4.7H_2O$ solution was prepared with ethanol as the solvent. A series of solutions were prepared by diluting 1 mM $FeSO_4.7H_2O$ solution to concentrations of 100, 200, 400, 600, 800, and 1000 µM to create a calibration curve. Finally, 1000 µg/mL ascorbic acid solution was prepared using water, and tea samples were diluted to a concentration of 0.5 mg/mL.

For the blank solution, 200 µL of water was added to a falcon tube. 200 µL of sample solution, ascorbic acid, $FeSO_4.7H_2O$, and blank solution were taken and placed in falcon tubes then, 1.8 milliliters of FRAP agent was added to all and vortexed. It was incubated in a 37 °C water bath for 30 minutes and then allowed to cool to room temperature. Absorbances are read at 593 nm against the blank solution using a UV-Vis spectrophotometer. The difference between the absorbance of the sample and the blank was calculated to observe the FRAP value. The results were expressed in mM equivalents of $FeSO_4$ per milligram and compared against the standard solution which is ascorbic acid. All measurements were carried out in triplicate.

DPPH Radical Scavenging Activity Method

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) method is based on the reaction of the DPPH radical with an antioxidant and the change in concentration, and the antioxidant activity is measured according to this change.[18] To observe the change in concentration of DPPH radical, measurements must be taken spectrophotometrically at 517 nanometers.[19]

First, 0.1 mM DPPH solution was prepared using ethanol as solvent. Then, stock tea solutions were prepared by diluting the solutions of tea samples to 0.5 mg/mL. Each tea solution was diluted with water to concentrations of 50, 100, 200, 300, and 500 µg/mL. Subsequently, 100 µg/mL ascorbic acid solution was prepared with water to be used as a control and diluted with water to concentrations of 2.5, 5, 10, 25, and 50 µg/mL. 1 mL of extract solvent was added to a falcon tube as a blank solution. Afterward, 2 mL of DPPH solution was added to every falcon tube. Each solution was vortexed for 30 seconds and then incubated in the dark for 30 minutes. Finally, the absorbance of the solutions against the blank solution was read at 517 nm in a spectrophotometer and recorded. This procedure was repeated three times. The following formula was used to calculate the percentage of free radical scavenging activity:

$$\% \text{ Free radical scavenging activity} = (1 - A_{sample} / A_{control}) \times 100$$

In this formula $A_{control}$ stands for the absorbance of the control and A_{sample} stands for the absorbance of sample solutions. Antioxidant activity of tea extracts expressed as IC_{50} which is the concentration of a substance that is necessary to neutralize 50% of the initial DPPH radical species. The results were compared with the control group, ascorbic acid.

Ferrous Ion Chelating (FIC) Method

3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine p,p'-disulfonic acid, namely ferrozine, is a red-colored structure that can chelate with Fe^{2+}, and the red color is reduced when ferrozine is chelated with other substances, such as antioxidants, and the method is based on this color change.[20] In this method, absorbance is measured spectrophotometrically at 562 nm and the results are calculated as percent inhibition.[21]

Firstly, solutions of tea samples were prepared and diluted to 0.5 mg/mL. Then, 0.5 mg/mL Na_2EDTA solution and 0.1mM $FeSO_4.7H_2O$ solution were prepared with water. 0.25 mM ferrozine solution was prepared using water as solvent. Then, 0.5 mL of extract solvent, 0.5 mL of $FeSO_4.7H_2O$ solution, and 1 mL of ferrozine solution were added to a falcon tube to prepare the blank solution.

After these preparations were completed, 0.5 mL of the extract solution (triplicate for each sample) and EDTA solution were added to the falcon tubes. 0.5 mL of 0.1 mM $FeSO_4.7H_2O$ and 1.0 mL of 0.25 mM ferrozine solution were added to each tube and vortexed. After vortexing, they were incubated for 10 minutes at room temperature in the dark. The absorbance values of the solutions in each tube were measured and recorded at 562 nm using a UV-Vis spectrophotometer. Finally, % inhibition was calculated with the formula:

$$\% \text{ inhibition} = (1 - A_{sample} / A_{control}) \times 100$$

Where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of samples. Higher % inhibition indicates high metal chelating ability.

Total Phenolic Content (TPC) Method

The total phenolic content (TPC) method is an antioxidant determination method that was obtained by making minor modifications to the Folin-Ciocalteu (FC) colorimetric method, using gallic acid as a standard and measuring the absorbance of the colored compound at 765 nm.[22]

Solutions of all tea samples were prepared and diluted to a concentration of 0.5 mg/mL. Then, the FC reagent was diluted with water in a ratio of 1:10. 7.5 % (w/v) Na_2CO_3 solution was prepared using water as the solvent. As the final solution preparation, 1000 µg/mL stock gallic acid solution was prepared with water and diluted to concentrations of 10, 20, 40, 60, 80, and 100 µg/mL. 0.4 mL of distilled water was used as a blank solution.

After adding 0.4 mL of extract and standard solutions to the falcon tubes, 2 mL of FC reagent was added, vortexed, and incubated for 5 minutes. 1.6 mL of 7.5% Na_2CO_3 solution was added and vortexed again. All tubes were incubated at room temperature for 1 hour.
Absorbances against blank solution at 765 nm were measured. The procedure was repeated three times and the results were expressed as gallic acid equivalent (GAE)/g of extract.

Statistical Analysis
Statistical analysis was performed by GraphPad Prism 9.3.1.

RESULTS AND DISCUSSION

Impact effect of Mixing Times and Solvents on Extract Yields
The data depicted in Table 1 and Table 2 represent the extract yield of six different forms of the tea plant using various mixing times and solvents.

As shown in Table 1 and Table 2, the highest yield for green leaves was obtained with 10 minutes of mixing time in ethanol. For the other samples, the highest yields were achieved using water, with mixing times of 60 minutes for S2, 20 minutes for S3 and S4, 10 minutes for S5, and 30 minutes for S6.

Table 1. Extraction data of tea samples using water as solvent

	10 min		20 min		30 min		40 min		60 min	
Sample (0.1 g)	Wt. of extract (g)	Avg. (mg)	Wt. of extract (g)	Avg. (mg)	Wt. of extract (g)	Avg. (mg)	Wt. of extract (g)	Avg. (mg)	Wt. of extract (g)	Avg. (mg)
Green leaves	0.0005	0.4333	0.0004	0.2333	-0.0010	-0.3333	-0.0028	-2.0333	0.0007	0.4000
	0.0004		0.0001		0.0003		-0.0015		-0.0002	
	0.0004		0.0002		-0.0003		-0.0018		0.0007	
Sample 2	0.0015	1.8333	0.0028	2.5000	0.0026	2.1667	0.0023	2.4000	0.0037	3.5667
	0.0019		0.0025		0.0029		0.0025		0.0035	
	0.0021		0.0022		0.0010		0.0024		0.0035	
Sample 3	0.0036	3.8333	0.0038	4.0667	0.0028	2.9000	0.0041	3.8000	0.0039	2.6667
	0.0029		0.0047		0.0028		0.0039		0.0032	
	0.005		0.0037		0.0031		0.0034		0.0009	
Sample 4	0.0037	4.0333	0.0049	4.2000	0.0027	3.3333	0.0034	3.5333	0.0012	2.9333
	0.0043		0.0039		0.0039		0.0038		0.0039	
	0.0041		0.0038		0.0034		0.0034		0.0037	
Sample 5	0.0038	3.9333	0.0015	2.0333	0.0033	2.9333	0.0020	2.2333	0.0029	2.9000
	0.004		0.0023		0.0029		0.0021		0.0024	
	0.004		0.0023		0.0026		0.0026		0.0034	
Sample 6	0.0044	4.6667	0.0036	4.6667	0.0053	5.3333	0.0045	3.2667	0.0040	4.3333
	0.0045		0.005		0.0053		0.0002		0.0042	
	0.0051		0.0055		0.0054		0.0051		0.0048	

Antioxidant Activity
Since plants have various bioactive compounds with high antioxidant activity, studies are carried out to clarify antioxidant activities for many plant species, thus revealing new antioxidant sources.[23] Phenolic compounds with antioxidant properties absorb and neutralize radicals, break down peroxides, and have redox properties.[24] In addition, phenolic compounds have a very important place among the compounds with antioxidant properties in many plants due to their stance against oxidative stress.[25] Structurally, plant phenolics are aromatic and contain hydroxyl groups, therefore they act as weak acids.[26]

The antioxidant effect of phenolic compounds is seen as hydrogen atom transfer, single electron transfer, loss of proton consecutively, and transition metal chelation.[27]

Oxidative stress can cause various diseases such as cancer, dementia, and asthma; however, plants with antioxidant properties can be used to treat and prevent these diseases.[28]

In this study, five different methods were used to measure the antioxidant activity of tea samples, using ethanol extract of green leaves and water extracts of S2, S3, S4, S5, and S6. (Table 3). CUPRAC is the first technique among them.

After the necessary procedure was carried out and the results were expressed as mM ascorbic acid equivalent per extract, it was seen that the sample with the highest CUPRAC activity was sample 6, while the sample with the lowest activity was green leaves. Sample 6 was followed by sample 3 and there was very little difference between samples 2 and 4. Sample 5 was the sample with the lowest activity after green leaves (Table 3).

Table 2. Extraction data of tea samples using ethanol as solvent

	10 min		20 min		30 min		40 min		60 min	
Sample (0.1 g)	Wt. of extract (g)	Avg. (mg)	Wt. of extract (g)	Avg. (mg)	Wt. of extract (g)	Avg. (mg)	Wt. of extract (g)	Avg. (mg)	Wt. of extract (g)	Avg. (mg)
Green leaves	0.0004		0.0003		-0.0013		0.0005		-0.0028	
	0.0013	0.933	0.0002	0.2333	-0.0011	-1.0333	0.0004	0.4000	-0.0009	-1.5333
	0.0011		0.0002		-0.0007		0.0003		-0.0009	
Sample 2	-0.0006		-0.0003		-0.0014		-0.0010		0	
	0.0002	-0.133	0.0007	0.2333	-0.0001	-1.0667	-0.0012	-0.7000	0	-0.0667
	0		0.0003		-0.0017		0.0001		-0.0002	
Sample 3	-0.0007		0.0004		-0.0001		-0.0004		-0.0013	
	-0.0004	-0.500	0.0005	0.6333	-0.0005	-0.5333	-0.0005	-0.5333	-0.0008	-0.6333
	-0.0004		0.0010		-0.0010		-0.0007		0.0002	
Sample 4	0.0002		0.0004		-0.0005		-0.0009		-0.0004	
	0.0008	0.4667	0.0003	0.4000	-0.0005	-0.3000	-0.0004	-0.9667	-0.0007	-0.7000
	0.0004		0.00 05		0.0001		-0.0016		-0.0010	
Sample 5	0.0005		-0.0501		-0.0007		-0.0008		-0.0018	
	0	0.1667	-0.0004	-17.066	-0.0012	-1.2333	-0.0001	-0.3000	-0.0008	-1.1333
	0		-0.0007		-0.0018		0		-0.0008	
Sample 6	0.0001		0.0003		-0.0003		-0.0002		-0.0007	
	0.0011	0.6333	0.0004	-0.1000	-0.0004	-0.4667	-0.0002	-0.4344	-0.0017	-1.2333
	0.0007		-0.0010		-0.0007		-0.0009		-0.0013	

Table 3. Antioxidant activity data of each sample

	CUPRAC (mM_{AAE}/g)	FRAP ($mM_{Fe(II)}/g$)	DPPH, $IC_{50}(\mu g/mL)$	FIC (mg_{EDTAE}/g)	TPC (mg_{GAE} /g)
Green leaves	763.17±2.93	475.43±8.10	463.25±4.10	17.95±7.39	14.10±3.70
Sample 2	1998.35±1.53	1211.89±3.97	213.47±7.47	28.76±4.43	40.09±2.66
Sample 3	2427.85±1.02	1638.83±1.04	135.59±0.35	14.21±1.44	65.62±4.29
Sample 4	2008.90±4.50	1147.85±5.66	313.78±3.83	34.48±4.92	67.08±3.08
Sample 5	1524.32±0.54	1042.09±7.67	214.31±9.70	23.92±1.36	45.60±3.28
Sample 6	2873.76±4.25	1446.70±7.23	133.29±8.58	31.71±5.12	74.39±1.69
Ascorbic acid	-	1800.67±1.08	71.64±10.30	-	-
Na_2EDTA	-	-	-	92.05±1.68	-

GAE: Gallic Acid Equivalent, AAE: Ascorbic Acid Equivalent, and EDTAE: EDTA Equivalent

* Each value is the mean of three experiments

* Sample 2 corresponds to the state of the tea leaves after the first process, and each sample is the state after the next process. Sample 6 is the ready-to-drink dry tea

The results of the FRAP method were expressed as mM equivalents of $FeSO_4$ per milligram and compared with ascorbic acid. As with the previous method, the sample with the lowest activity is green leaves. Similarly, the samples with the highest activity are samples 3 and 6. The difference between samples 4 and 2 is again very small. Sample 5 is the sample with the lowest activity after green leaves, although it is close to 2 and 4.

The third method, the DPPH method, was carried out and the antioxidant activity of tea extracts was stated as IC_{50}. The results were compared with the control, ascorbic acid. According to the data of this method, the tea sample with the least antioxidant properties is green leaves and there is a significant difference between it and the other samples. The sample with the most antioxidant properties is sample 6, followed by sample 3. After sample 3, samples 2, 5, and 4 come in order.

The FIC method was conducted as the fourth method and the results are calculated as percent inhibition. EDTA solution was used as the control group. The sample with the lowest activity is sample 3, but the green leaves were very close to it. The sample with the highest activity is sample 4, and they have values very close to sample 6. Sample 6 is followed by samples 2 and 5.

In the last method, TPC, the results were expressed as gallic acid equivalent (GAE)/g of extract. When the samples were compared, it was seen that the sample with the lowest antioxidant activity was green leaves and there was a big difference between this sample and the other samples. The sample with the most antioxidant properties was sample 6, followed by samples 4 and 3.

According to the experimental studies, three out of five methods showed that sample 6 was the tea sample with the highest antioxidant properties. Unprocessed green leaves ranked last in terms of antioxidant properties in the majority of the methods. Throughout the production process of black tea, a significant amount of catechins is oxidized and partially polymerized due to enzymatic reactions, leading to the creation of secondary phenolic compounds such as theaflavins and thearubigins.[29] Consistent with this, the findings of this study revealed that Sample 6, representing the final stage of black tea processing, displayed the highest antioxidant activity among all samples. In terms of antioxidant activity, Sample 2 ranked third in three methods, while Sample 5 ranked fourth in three methods.

Green tea is a type of tea that demonstrates higher antioxidant activity compared to black tea.[30] In this study, the green leaves were identified as the sample with the least antioxidant activity. If green tea leaves and black tea leaves were compared, the green tea leaves would have yielded better results. However, the focus of this study was to determine the stage of black tea that exhibited the highest antioxidant activity.

Antioxidant activity was found to be highly significant ($P < .0001$) with DPPH, FRAP, and FIC techniques for all samples when compared with ascorbic acid for FRAP and DPPH and with Na2EDTA for FIC test (Figure 2). There is a significant correlation between the five methods of antioxidant activity at $P < .0001$ (Figure 3).

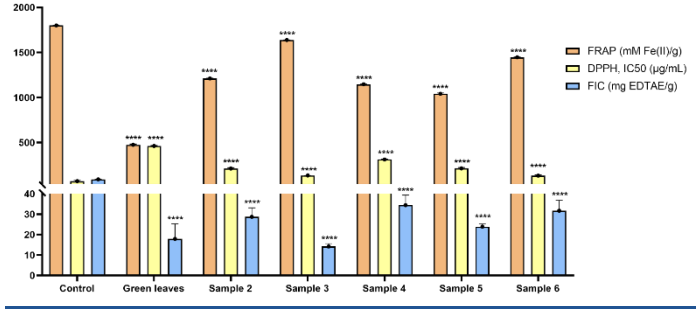

Figure 2. Comparison of antioxidant activity of Turkish black tea at various processing stages, namely green leaves, sample 2, sample 3, sample 4, sample 5, and sample 6. The antioxidant potential of the samples compared with ascorbic acid for FRAP and DPPH; and Na_2EDTA for the FIC test, data are represented as ±SE, N = 3. Statistical analysis is two-way ANOVA, ****$P < .0001$

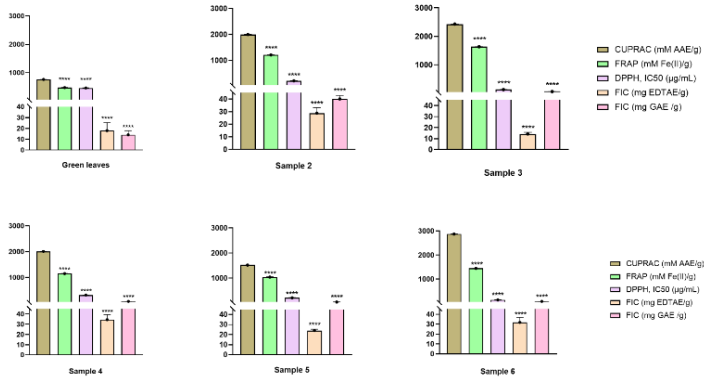

Figure 3. Comparison of antioxidant potential of Turkish black tea at various processing stages (A) green leaves, (B) sample 2, (C) sample 3, (D) sample 4, (E) sample 5, and (F) sample 6 by five different techniques, data are represented as ±SE, N = 3. Statistical analysis is one-way ANOVA ****$P < .0001$

CONCLUSION

Natural antioxidants have become the center of attention after studies emerged about the possible negative effects of consuming synthetic antioxidants. Tea is one of the three most consumed beverages in the world, and it is estimated that catechins, a type of polyphenol, provide antioxidant properties to tea and play a role in breaking down radicals such as lipid alkoxyl and peroxyl. In this study, five different spectrophotometric methods were used to measure the antioxidant activity at each processing stage of black tea. The results were largely consistent with each other. The main aim of the study was to identify the precise stage at which antioxidant activity attains its maximum level. The findings are intended to guide consumers in selecting the optimal sample for tea preparation, thereby enhancing the intake of antioxidants and promoting health benefits. The findings derived from this research indicate that, when evaluating the antioxidant properties of black tea, the samples exhibiting the most pronounced antioxidant capabilities demonstrated statistically significant results in comparison to the standards.

Nevertheless, care should be taken to use the correct amount, as excessive antioxidant intake can disrupt the redox balance in the body. For this reason, people should not only consider black tea as a natural antioxidant source but also should examine the antioxidant content of the foods they consume in their daily lives. This research will provide valuable insights for individuals who regularly drink black tea and explore herbal approaches for health maintenance, disease prevention, and overall wellness.

Ethics Committee Approval: Ethical approval and informed consent are not required in our study as no research was conducted on human or animal specimens.

Author Contributions: Concept – M.Ş, S.G; Design - M.Ş, S.G; Supervision - M.Ş; Resources – S.G; Materials - M.Ş, S.G; Data Collection and/or Processing - S.G, İ.A.K; Analysis and/or Interpretation - M.Ş, S.G, İ.A.K; Literature Search - M.Ş, S.G; Writing Manuscript -; Critical Review - M.Ş

Declaration of Interests: The authors have no conflicts of interest to declare.

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Synthesis, Characterization and Docking Studies of a Schiff Base Ligand and Some Metal Complexes

Ferit OKTAY[1]

Sümeyra Tuna YILDIRIM[2]

[1]Department of Pharmaceutical Sciences, Institute of Health Sciences, Erzincan Binali Yıldırım University, Erzincan, Türkiye

[2]Department of Analytical Chemistry, Faculty of Pharmacy, Erzincan Binali Yıldırım University, Erzincan, Türkiye

ABSTRACT

Objective: To synthesize a Schiff base ligand and some metal complexes, perform characterization studies and elucidate the action mechanisms of the synthesized compounds with the Schrödinger Suite computer aided molecular modeling program.

Methods: A Schiff base ligand has been synthesized by the reaction of a salicylaldehyde derivate with an aminophenol. With using this ligand, metal complexes were prepared from Co(II), Ni(II), Cu(II) and Zn(II) acetate salts. The structures of the synthesized compounds were confirmed by different spectroscopic and microscopic techniques such as Elemental Analysis, FT-IR, ^{1}H-NMR, ^{13}C-NMR, UV-Vis, XRD, SEM and TGA. In order to elucidate the mechanism of action of the synthesized compounds, some descriptive molecular properties were calculated using the Schrödinger Suite computer aided molecular modeling program.

Results: Within the scope of the study, a ligand and its complexes were synthesized. The structures of compounds were elucidated and molecular docking studies showed that Zn(II) complex had the highest scores obtained.

Conclusion: Structural characterization showed that ligand at the metal complexes act as bidentate chelates by binding to the metal ion from the imine nitrogen and phenolic oxygen. The mechanism of action of the synthesized compounds and the active site where the coupling will take place were determined, and the interactions of possible drug molecule candidates synthesized with the target receptor site were calculated.

Keywords: Ligand, Metal Complex, Molecular Docking, Schiff Base, Spectroscopic Characterization

INTRODUCTION

The chemistry of coordination compounds is well known and widely studied because of many applications in different areas. This is due to the chelates they make with metal ions. An organic compound can be converted into inorganic compound as a complex with a transition metal. The applications of the metal complexes in qualitative and quantitative chemical analysis have also been the issue of many studies.[1, 2] Schiff bases are acknowledged condensation products in organic and inorganic chemistry.[3, 4]

These compounds are the class of ligands that are used frequently because of their useful chelates due to their easy synthesis, structural diversity, electronic control mechanisms, and ability to coordinate with transition metals. These compounds having several recourses in many fields such as organic chemistry, inorganic chemistry and also as pharmaceutical targets[5, 6] and they include a variety of therapeutically powerful applications in medicinal chemistry.[7] These ligands were synthesized from the condensation of an amino group with a carbonyl compound. Generally, nitrogen, oxygen and sulphur donor atoms are involved in the coordination of metals in the active sites of numerous metallo-biomolecules. In the literature, it has been reported that the binding of the drug to a metallo element increases its activity and in some case the complex has more curative properties than the parent drug.[8, 9] In addition to that, they used for purposes such as catalytic and enzymatic reactions, electronics, polymer industry, luminescence materials, magnetism, and molecular design.[10, 11]

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Corresponding author:
Sümeyra TUNA YILDIRIM
E-mail: stuna@erzincan.edu.tr

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In this study, the synthesis of a ligand and its complexes with Co(II), Ni(II), Cu(II) and Zn(II) acetate salts were performed and then the structures of the synthesized compounds characterized by elemental analysis, magnetic susceptibility, IR, ^{1}H-NMR, ^{13}C-NMR, UV-Vis, XRD, SEM, TGA methods. Molecular docking studies showed that the activity of the Zn (II) complex was higher.

METHODS

Experimental Materials and Instruments

o-Aminophenol (99%, Sigma-Aldrich), cobalt(II) acetate tetrahydrate (99%, Merck), 5-fluorosalicylaldehyde (97%, Sigma-Aldrich), nickel(II) acetate tetrahydrate (99.9%, Sigma-Aldrich), copper(II) acetate monohydrate (99%, Merck), zinc(II) acetate dihydrate (99.5%, Merck), *p*-toluenesulfonic acid (98%, Merck) and acetone, ethanol, methanol, diethyl ether, dimetilformamide, dimethyl sulfoxide were used as solvents at analytical reagent grade and provided from Merck Company.

Infrared spectra was recorded on a Nicolet 6700 FT-IR Thermo Scientific spectrophotometer. The NMR spectra of compounds were recorded at room temperature using a Bruker DPX-400 spectrophotometer. Melting points were determined with a Buchi B-540 digital apparatus. The UV–Vis spectra of the compounds were taken a Shimadzu 1240 model UV-Vis spectrophotometer. The elemental analyses for C, H and N were obtained from the LECO-932 CHNSO model elemental analyzer. The X-ray diffraction data of ligand was recorded using a Panalytical Empyrean diffractometer with a scan rate of 2 min^{-1} in the 2θ range of 10–80˚. Morphological study of ligand (SEM with EDAX) was carried out using the Scanning Electron Microscope Fei Quanta FEG 450 instrument. Schrödinger Suite computer-aided molecular modeling program was used to elucidate the action mechanisms of the synthesized compounds.

Synthesis of LH Ligand

o-Aminophenol (1.09 g, 10 mmol) and (0.01 g) *p*-toluene sulphonic acid were dissolved in 20 mL absolute ethyl alcohol and slowly dropped onto the solution of 5-fluorosalicylaldehyde (1.40 g, 10 mmol) in 20 mL absolute ethyl alcohol. This solution was reflux at 60 °C for 3 hours. The pomegranate flower color product obtained as a result of the reaction was filtered off, washed several times with hot distilled water and diethyl ether, recrystallized from ethanol and dried at room temperature.[12, 13] The synthesis of Schiff base ligand is shown in Figure 1.

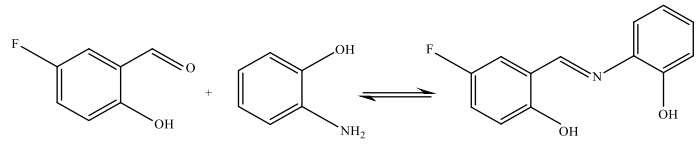

Figure 1. Synthesis of (E)-2-(((2-hydroxyphenyl)imino)methyl)-4-fluorophenol

Synthesis of Metal Complexes

The synthesized ligand (0.46 g, 1.00 mmol) was dissolved in 10 mL absolute ethyl alcohol and released into a 100 mL flask in the parallel synthesis device, and metal acetates [cobalt(II) acetatetrahydrate (0.125 g, 0.50 mmol), nickel(II) acetatetrahydrate (0.124 g, 0.50 mmol), copper(II) acetate monohydrate (0.10 g, 0.50 mmol), zinc(II) acetate dihydrate (0.110 g, 0.50 mmol)] were mixed dropwise with a dropping funnel. The reaction mixture was refluxed for 2-4 hours. After the synthesized products were rested, they were washed with hot pure water, ethyl alcohol and diethyl ether and dried under vacuum. [12, 13] The synthesis of Schiff base complexes is shown in Figure 2.

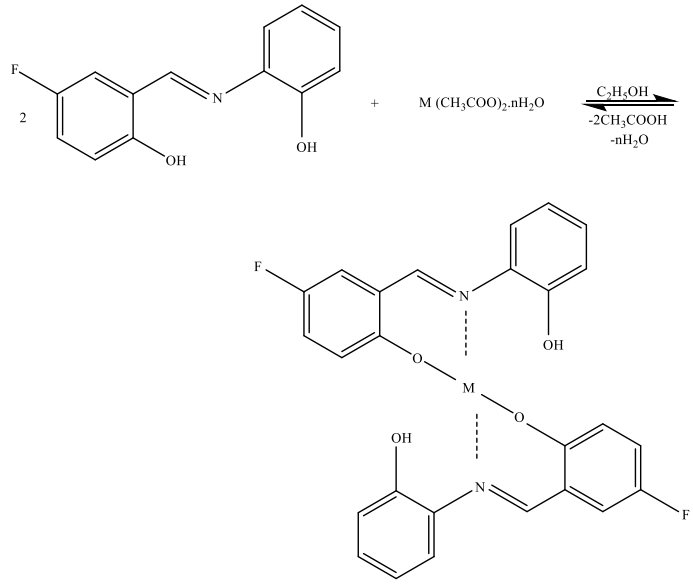

Figure 2. Synthesis of metal complexes of LH Ligand. [M: Co(II), Ni(II), Cu(II), Zn(II)]

RESULTS AND DISCUSSION

Structural Characterization

The stoichiometric ratio of ligand with Co^{+2}, Ni^{+2}, Cu^{+2}, Zn^{+2} structural characterization were investigated by various instrumental analysis methods. As can be seen in Table 1, the metal: ligand ratio is 1:2 in the elemental analysis of the complexes of ligand. [14, 15] Some analytical and physical data of the synthesized compounds were given in Table 1.

Table 1. Analytical and Physical Data of Ligand and Metal Complexes

Formula	W (g/mol)	Colour	m.p (°C)	μ_{eff}	Yield (%)	Elemental Analysis, % Calculated (Found)		
						C	H	N
$C_{13}H_{10}NO_2F$	231.226	Pomegranate Flower	118-120	-	89.00	67.53 (67.29)	4.36 (4.29)	6.06 (6.06)
$CoC_{26}H_{18}N_2O_4F_2$	519.369	Dark Brown	> 300	4.19	56.00	60.13 (60.02)	3.49 (3.36)	5.39 (5.21)
$NiC_{26}H_{18}N_2O_4F_2$	519.129	Light Green	> 300	2.62	48.00	60.16 (60.07)	3.50 (3.27)	5.40 (5.36)
$CuC_{26}H_{18}N_2O_4F_2$	523.982	Dark Green	> 300	1.86	62.00	59.60 (59.86)	3.46 (3.48)	5.35 (5.19)
$ZnC_{26}H_{18}N_2O_4F_2$	525.826	Light Yellow	> 300	Dia.	66.00	59.39 (59.27)	3.45 (3.37)	5.33 (5.28)

IR spectra of the LH ligand and Co(II), Ni(II), Cu(II), Zn(II) complexes taken in KBr were examined. The stretching vibration of the phenolic O-H group was seen as a flat peak at 3442 cm^{-1} at LH ligand IR spectrum (Figure 3). A sharp peak was observed at 1632 cm^{-1}, which indicates the presence of imine group in the structure. The aromatic C-H weak peak was at 3045 cm^{-1}, the aliphatic C-H stretching vibration was at 2965-2920 cm^{-1}, the C=C stretching vibration was at 1500 cm^{-1}-1440 cm^{-1} and the phenolic C-O stretching vibration was at 1282 cm^{-1} at the spectrum. In addition, the disappearance of the peak at 1656 cm^{-1} belonging to –C=O stretching vibration of 5-fluorosalicylaldehyde, which was the starting material and the formation of a peak belonging to –C=N stretching vibration instead of this peak, also was supported the completion of the reaction.[16, 17]

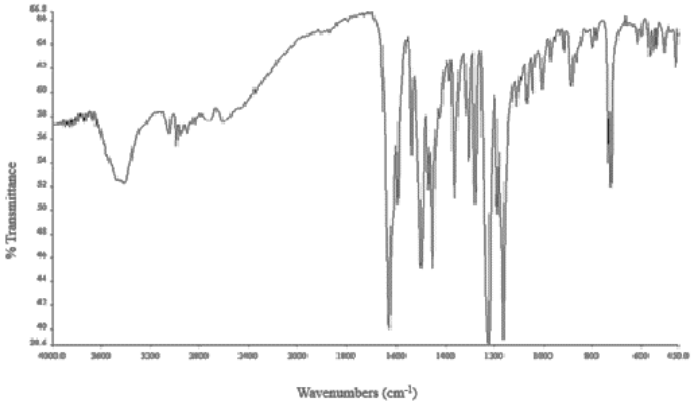

Figure 3. IR Spectrum of LH Ligand

Some changes were observed in the stress vibration of the –C=N group and the bending vibration of the phenolic O-H group at IR spectra metal complexes. The characteristic -C=N stretch vibration, which shows the presence of imine in the ligand and observed at 1632 cm^{-1}, shifted to the low frequency region of 1608-1617 cm^{-1}. This shift can be explained by the coordination of the nitrogen atom in the azomethine group with the metal during the complex formation. In other words, the nitrogen atom is in coordination by giving its unshared electrons to the metal ion.

It also shows that in the shift in the O-H peak in the ligand, the phenolic O-H is in coordination with the metal ion by throwing its proton. In addition, complex structures, which are observed at 1282 cm^{-1} and are characteristic band for phenolic C-O stress vibration, showed slippage. This shift supports that the protonated phenolic oxygen enters into coordination with metal ions during complex formation. Like in the ligand, the O-H stretch vibration in the complexes is in the form of a flat peak in the region of 3400-3450 cm^{-1}.[16, 17]

A proton singlet at 14.25 ppm was observed in the ^{1}H-NMR spectrum of LH ligand and belongs to the proton of the phenolic OH group. The chemical shift observed as a single proton singlet at 9.04 ppm in the spectrum of H proton of the –CH=N group in the structure. Protons belongs to the aromatic ring were observed as multiplets in the range of 6.89-7.48 ppm. Considering the integral ratios, the proton numbers were in harmony with the predicted structure. In addition, the chemical shift of the OH group proton was observed at 9.98 ppm.[18, 19]

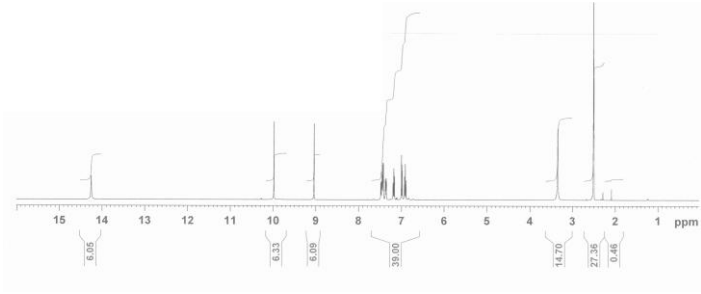

Figure 4. ^{1}H-NMR Spectrum of LH Ligand

The ^{13}C-NMR spectrum of LH ligand (Figure 5) taken in the mixture of $CDCl_3$ and DMSO-d_6 was examined the signal of the carbon atom in the imine bond was resonated at 162.03 ppm and chemical shift of the C-OH carbon was observed at 159.80 ppm. Chemical shift of C-N group carbon was observed at 134.85 ppm and the peaks for the aromatic ring carbons were observed in the range of 119.59-132.95 ppm.[18, 20]

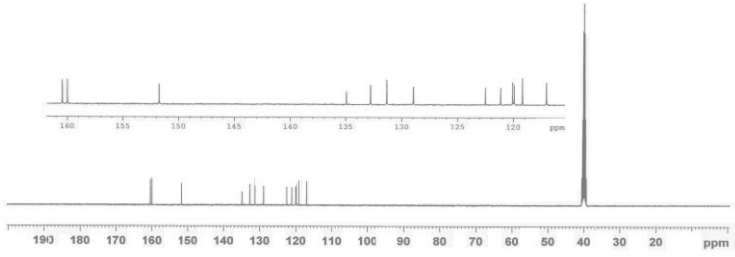

Figure 5. ^{13}C-NMR Spectrum of LH Ligand

The geometries of metal complexes were determined from the magnetic susceptibility measurements of the LH ligand of Co^{+2}, Ni^{+2}, Cu^{+2} and Zn^{+2} complexes. The measured magnetic susceptibility values show that the Co^{+2}, Ni^{+2} and Cu^{+2} complexes were paramagnetic and the Zn^{+2} complex was diamagnetic.[21, 22] The measured μ_{eff} value for the Co^{+2} complex was 4.09 B.M. and corresponds to 3 electrons. This value shows us that the Co (II) complex with the d^7 configuration was in tetrahedral geometry. The μ_{eff} value measured for the Ni^{+2} complex was 2.71 B.M. and corresponds to 2 electrons. This value shows us that the Ni (II) complex was also in tetrahedral geometry. For the Cu^{+2} complex, the μ_{eff} value corresponds to 1.70 B.M. and corresponds to 1 electron. However, it is not possible to determine whether the Cu (II) complex was tetrahedral or square. The diamagnetic Zn (II) complex prefers the tetrahedral structure because it has d^{10} configuration.

In the UV-Vis spectrum of the LH ligand (Figure 6), a band is observed at 290 nm due to the π→π* electronic transition, while the n→π* transition is observed as a broad peak at 360 nm. In the complexes, this band, which was observed at 290 nm and belongs to the π→π* electronic transition, observed at approximately the same location. The band originating from the n→π* transition of the unpaired electrons of the nitrogen atoms of the imine group has shifted to the wavelength of 380-420 nm in the complexes. This supports the coordination between the imine group and the metal ions.[23]

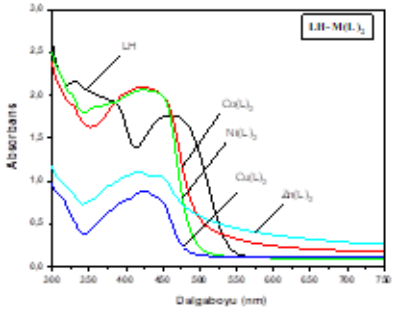

Figure 6. UV-Vis. Spectrum of LH Ligand

XRD patterns of the Schiff base ligand was obtained at room temperature with using CuKα1 λ=1.5405 Å in the range 2θ=10–100°, operated at 45kV and 40 mA. As seen in Figure 7, Schiff base ligand exhibited sharp crystalline peaks. The obtained diffractogram was put out the ligand was acknowledged by two important characteristic peaks appearing at 2θ=19.28˚and 22.54˚, due to imine group. The XRD pattern of ligand was showed a semicrystalline nature.[24, 25]

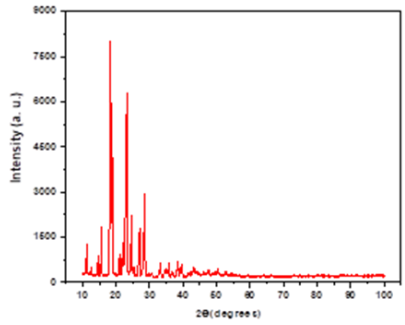

Figure 7. XRD Pattern of LH Ligand

The surface morphology of the ligand was investigated using SEM analysis and the ligand had a compact and dense surface morphology (Figure 8). Also, ligand had nanorod structure and localized clusters on the surface morphology.[25,26]

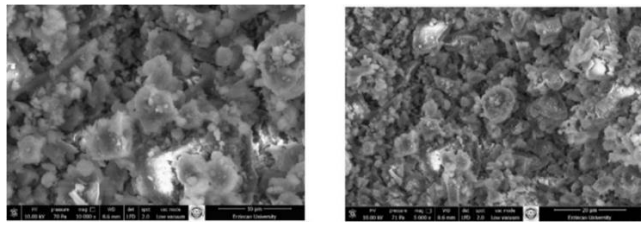

Figure 8. SEM of LH Ligand

Schrödinger Suite computer-aided molecular modeling program was used to elucidate the mechanism of action of the synthesized compounds. The active site where the docking will occur was determined and the interactions of possible drug molecule candidates synthesized by the Structure Based Drug Design method with the target receptor site were calculated using the Glide module in the Schrödinger program package. The ZnL_2-CA I (PDB ID: 6F3B) interaction diagram and ZnL_2-CA II (PDB ID: 6H34) interaction diagram obtained as a result of molecular docking were given at Figure 9 and Figure 10.[27]

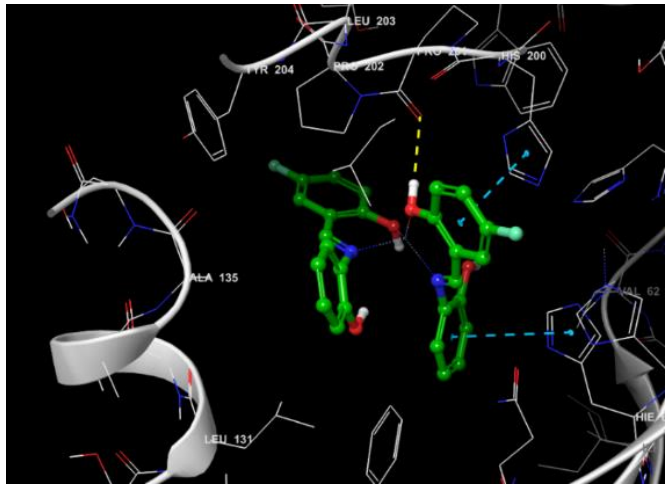

Figure 9. Interaction Diagram of ZnL_2-CA I (PDB ID: 6F3B)
(Obtained as a Result of Molecular Docking of Schiff Base Ligand with Metal Complexes)

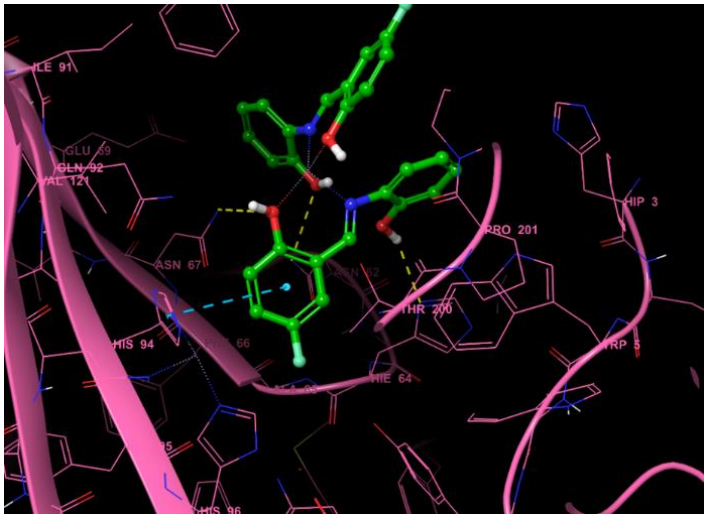

Figure 10. Interaction Diagram of ZnL_2-CA II (PDB ID: 6H34)
(Obtained as a Result of Molecular Docking of Schiff Base Ligand with Metal Complexes)

CONCLUSION

In conclusion, Schiff base ligand, (E)-2-(((2-hydroxyphenyl)imino)methyl)4-fluorophenol, was synthesized from the reaction of 5-fluorosalicylaldehyde and *o*-aminophenol. The metal complexes were prepared from the reaction with metal acetates. The structures of Schiff base ligand and metal complexes obtained were elucidated by using elemental analysis, IR, ^{1}H-NMR, ^{13}C-NMR, UV-Vis, XRD, SEM, magnetic susceptibility and thermogravimetric analysis techniques. It was determined that the metal/ligand ratio was 1:2 in all complexes and that Schiff base ligand was bonded to the metal atom with the nitrogen atom in the imine and the phenolic oxygen atom. As a result of molecular docking of CA I and CA II enzymes with the synthesized Schiff base ligands and complexes, it was determined that the Zn (II) complex had the highest scores obtained.

Ethics Committee Approval: Ethical approval and informed consent are not required in our study as no research was conducted on human or animal specimens.
Peer-review: Externally peer-reviewed.
Author Contributions: F.O. and S.T.Y. contributed equally. Concept, Design, Supervision, Resources, Data Collection, Literature Search, Synthesis, Characterization, Writing Manuscript was carried out by both authors. All authors have read and agree to the published version of the manuscript.
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Development and *in vitro* Characterization of Nanoemulsion and Nanoemulsion-based Gel Formulations Containing *Heracleum persicum* Ethanol Extract

Soheil POURAKBAR FARZAMİ[1]
Afife Büşra UĞUR KAPLAN[2]
Yaşar Furkan KILINBOZ[3]
Meltem ÇETİN[2*]

[1]Atatürk University, Faculty of Pharmacy, Erzurum, Türkiye
[2]Atatürk University, Faculty of Pharmacy,Department of Pharmaceutical Technology, Erzurum, Türkiye
[3]Ondokuz Mayıs University, Faculty of Pharmacy, Department of Pharmaceutical Technology, Samsun, Türkiye

ABSTRACT

Objective: We aimed to prepare the ethanol extract (Hp-Et) of the aerial parts of *Heracleum persicum* (Hp) and to determine its antioxidant capacity. We also aimed to develop nanoemulsion (NE) and NE-based gel (NEG) formulations containing this extract for topical application to the skin for wound healing and to characterize these formulations *in vitro*.

Methods: After the preparation of Hp-Et, its antioxidant capacity was determined by FRAP, CUPRAC, and DPPH methods. Then, blank NE (B-NE) and the extract-containing NE (Hp-Et-NE) formulations were developed and *in vitro* characterized [morphological analysis; centrifuge test; the determination of droplet size (DtS), polydispersity index (PDI) and zeta potential; viscosity and pH measurements; FT-IR analysis]. Additionally, B-NEG and Hp-Et-NEG were prepared and *in vitro* characterized [viscosity and pH measurements; FT-IR analysis].

Results: Droplet size and zeta potential values of NE formulations were around 200 nm and -30 mV, respectively. PDI values were less than 0.4. The pH values for NE and NEG formulations were in the range of 4.63±0.01-5.73±0.01. The NE and NEG formulations showed Newtonian and pseudoplastic behaviors, respectively.

Conclusion: Hp-Et-NEG exhibits the desired pseudoplastic behavior for topical application to the skin.

Keywords: Ethanol extract, *Heracleum persicum*, *in vitro* characterization, nanoemulsion, nanoemulsion-based gel

INTRODUCTION

Nanoemulsions (NEs), which consist of an oil phase, aqueous phase, and emulsifier/s and have a generally accepted droplet size (DtS) range of 20-200 nm, are also known as mini-emulsions and submicron emulsions and have long-term physical stability. NEs' DtS affects their stability and optical and rheological properties. It also affects the absorption/penetration of active ingredients.[1,2] Although NEs have various advantages, including increasing the solubility of low water-soluble active ingredients and improving their bioavailability, they also have disadvantages, including low retention time and spreadability due to their low viscosity. Therefore, by using a suitable gelling agent (such as Carbopol, chitosan, sodium carboxymethylcellulose), the NE is converted into a NE-based gel (nanoemulgel, NEG) and turned into a system that can be easily applied to the skin topically.[3] In recent years, studies have been carried out to prepare NE-based gel formulations for wound healing. Morsy et al.[4] prepared a NE-based gel formulation containing atorvastatin for topical application for wound healing and reported that the prepared formulation provided significant wound healing. In another study, they reported that the NE-based gel formulation containing curcumin prepared for wound healing played an important role in increasing the skin penetration and wound-healing activity of curcumin.[5]

Heracleum persicum (Hp), belongs to the family Apiaceae, is a perennial plant and widely used as a flavoring, carminative, digestive, and spice. It is a native plant in Türkiye (registered in the upper Euphrates, upper Murat-Van, Hakkari, and Adana sections).[6–8]

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Corresponding author: Meltem Çetin
E-mail: melcetin@atauni.edu.tr

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Hp has various biological effects, including antimicrobial, anti-inflammatory, analgesic and antioxidant properties. In traditional medicine, this plant contains bioactive compounds such as flavonoids, furanocoumarins, triterpenes, terpenoids, anethole, and alkaloids. Moreover, it has been used for the treatment of urinary, respiratory, neurological, rheumatological, and gastrointestinal disorders.[6,7] Synergistic treatments with anti-inflammatory, antibacterial, and antioxidant, properties should be developed to promote wound healing.[9] According to traditional Persian medicine, Hp is among the plants known to have wound-healing properties. However, the wound-healing properties of Hp have not been investigated in modern research.[10]

In our study, we aimed to prepare the ethanol extract of the aerial parts of Hp (Hp-Et) and to determine its antioxidant capacity by "ferric reducing antioxidant power (FRAP)", "cupric reducing antioxidant capacity (CUPRAC)", and "DPPH radical scavenging" assays. We also aimed to develop NE and NEG formulations containing this extract for topical application to the skin for wound healing and to characterize these formulations *in vitro*.

METHODS

Materials

St. John's wort oil, Labrafac PG, Lipoid S100, Protasan UP G213 and Kolliphor RH 40 were obtained from Pharmaoils (Türkiye), Gattefossé (France), Lipoid GmbH (Germany), Novamatrix (Norway), and Sigma (USA), respectively. Ethanol, $CuCl_2.2H_2O$, diphenylpicrylhydrazyl (DPPH), tripyridyltriazine (TPTZ), neocuproine (Nc), ammonium acetate, and $FeCl_3$ were obtained from Tekkim (Türkiye), Merck Millipore (Germany), and Sigma-Aldrich (USA), respectively.

Hp-Et's Preparation and Its Antioxidant Capacity Determination

After pulverizing the dried aerial parts of Hp with a laboratory blender, we added 250 mL of ethanol to powder (10 g) and mixed it at room temperature on a magnetic stirrer (600 rpm; 24 h). After the filtration, ethanol was removed by a rotary evaporator (40 °C, 90 rpm). FRAP, CUPRAC, and DPPH radical scavenging activity assays were used to determine its antioxidant capacity.

In the FRAP assay, first, FRAP reagent was prepared freshly [0.3 N acetate buffer (pH 3.6), 10 mM TPTZ solution (in 40 mM HCl) and 20 mM $FeCl_3$ solution; 10:1:1 (v/v/v)].[11] Then, 10 µL of the sample (Trolox standard solution or Hp-Et) and 200 µL of FRAP reagent were added into the wells of the 96-well plate and incubated for 30 min at room temperature, protected from light. At the end of the period, absorbance was measured at 593 nm.[11–13]

In the CUPRAC assay, 10 mM $CuCl_2.2H_2O$ solution, 7.5 mM Nc solution and ammonium acetate (pH 7.0) buffer were prepared .[14] Then, 66 µL of the sample (Trolox standard solution or Hp-Et) was added into each well of 96-well plates containing $CuCl_2.2H_2O$ solution, Nc solution, and acetate buffer. After incubation (room temperature, protected from light; 30 min), absorbance was read at 450 nm.

In the DPPH radical scavenging assay, 70 µL of freshly prepared DPPH solution and 210 µL of the sample (Trolox standard solution or Hp-Et) were added into each well of 96-well plates. After 30 min incubation at room temperature, protected from light, absorbance measurements were carried out at 515 nm.[15] Then, the percent DPPH inhibition was calculated.[16]

NE and NEG Formulations' Preparation

A mixture of St. John's Wort oil and Lipoid S100 in a hot water bath (70 °C) was prepared and then mixed on a magnetic stirrer until it reached room temperature (750 rpm). Hp-Et (10 mg) was dissolved in Labrafac PG. Then, it was added to the oil phase and mixed it on a magnetic stirrer (750 rpm; 5 min). The aqueous phase (Kolliphor RH 40 and ultrapure water) was added to the prepared oil phase under magnetic stirring (750 rpm). Hp-Et-NE formulation was prepared by first applying high-speed mixing (Ultraturrax; 27500 rpm, 6 min) and then ultrasonication (65% power, 12 min) to reduce the DtS of the prepared coarse emulsion.

The B-NE formulation was prepared using the same procedure without adding Hp-Et. Furthermore, Protasan UP G213 (1%) was added to the NE formulations and mixed overnight on a magnetic stirrer (600 rpm) to prepare the B-NEG and Hp-Et-NEG formulations.

Characterization of NE and NEG Formulations

Centrifuge test

NE formulation (5 g) was centrifuged (15 min; 3500 rpm) to assess whether phase separation occurred.

Determination of DtS, polydispersity index (PDI), zeta potential, and pH values of formulations

The Zetasizer Nano ZSP ("Malvern Instruments Ltd., UK") was used to determine the DtS, PDI, and zeta potential values of 200-fold diluted NE formulations.

Additionally, pH measurements of NE and NEG formulations were performed using a pH meter ("Thermo Scientific, Orion 3 StarTM, USA").

FT-IR Analysis

FT-IR spectra of Hp-Et, B-NE, B-NEG, Hp-Et-NE, and Hp-Et-NEG were taken in the range of 4000-400 cm^{-1}.

Rheological analysis

Viscosity measurements of B-NE, B-NEG, Hp-Et-NE, and Hp-Et-NEG were carried out at room temperature using a Brookfield viscometer (RV DV2T; USA).

Statistical analysis

The "Independent t-test" ("SPSS Statistics Version 22.0 software; SPSS Inc., USA") was used to compare the obtained results. The results are shown as mean±standard deviation (SD) (P<.05: the difference was considered significant).

RESULTS AND DISCUSSION

FRAP, CUPRAC, and DPPH assays were used to determine the antioxidant capacity of Hp-Et. Trolox solution in ethanol was used as a standard for all three assays. Figure 1 shows the obtained *Trolox standard curves* and equations (in the concentration range of 1-100 µg/mL for FRAP and CUPRAC assays and 1-10 µg/mL for DPPH assay). We presented the total antioxidant capacity values of Hp-Et in Table 1.

In a study, antioxidant capacities of Hp Desf., *Chaerophyllum macropodum* Boiss., and *Prangos ferulacea* (L.) Lindl. were determined using DPPH assay. 50% inhibitory concentration values for *P. ferulacea, C. macropodum* and Hp were 0.242 mg/mL, 0.623 mg/mL and 0.438 mg/mL, respectively.[17]

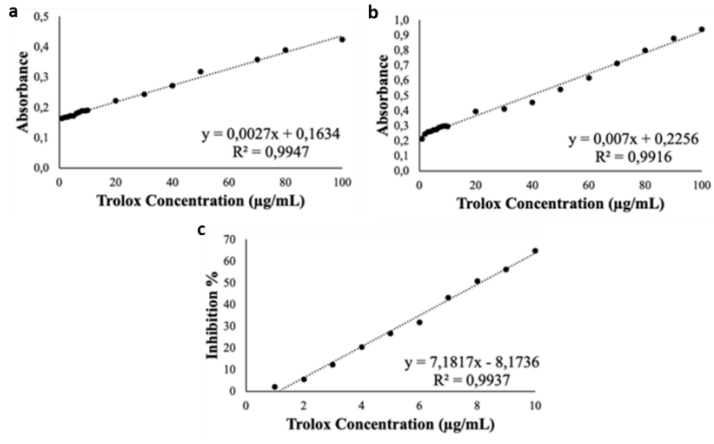

Figure 1. *Trolox standard curves* and equations for FRAP (a), CUPRAC (b) and DPPH (c) assays.

Table 1. Total antioxidant capacity values for Hp-Et (n=3; Mean±SD)

	Trolox-equivalent antioxidant capacity (µg/mL)		
	FRAP	CUPRAC	DPPH
Hp-Et (500 µg/mL)	18.44±0.11	24.25±0.58	12.60±0.08

After the centrifuge test, there was no creaming or phase separation in the B-NE and Hp-Et-NE formulations. Table 2 shows the DtS, PDI, and zeta potential values determined for B-NE and Hp-Et-NE formulations. DtS, PDI, and zeta potential affect the physical stability of NEs. The increased surface area (due to nano-sized droplets) also affects the skin penetration of active compounds.[18–20] The Brownian motions of nano-sized droplets are significant for the physical stability of NEs.[18,19]

The DtSs of the NE formulations prepared in our study were around 200 nm (Table 2). There was no statistically significant difference between the DtSs of the B-NE and Hp-Et-NE formulations (P>.05). The PDI values found for the B-NE and Hp-Et-NE formulations were less than 0.4 (Table 2). The PDI is usually less than 0.05 for highly monodisperse standards, while it is more than 0.7 for systems with broad size distribution. In this case, acceptable PDI values are in the range of 0.05-0.7, depending on the sample type.[21]

The zeta potential values of the NE formulations prepared in our study are around -30 mV (Table 2). There was no significant difference between the zeta potential values of the B-NE and Hp-Et-NE formulations (P>.05). For emulsions, zeta potential values of +30 mV and above and -30 mV and below are considered appropriate for good physical stability.[20] The TEM image of the Hp-Et-NE formulation is given in Figure 2. Nano-sized and nearly spherical droplets were obtained.

Table 2. The DtS, PDI and zeta potential values determined for B-NE and Hp-Et-NE formulations (Mean±SD; n=9)

Formulation	DtS (nm)	PDI	Zeta Potential (mV)
B-NE	204.01±11.40	0.368±0.027	-33.14±0.96
Hp-Et-NE	210.86±8.55	0.370±0.031	-33.44±1.25

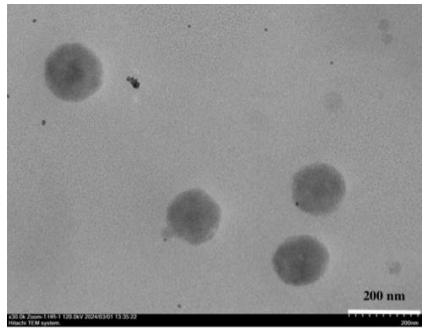

Figure 2. The TEM image of the Hp-Et-NE formulation

In addition, the pH values measured for NE and NEG formulations are given in Table 3. Human skin pH is generally acidic but can vary widely between pH 4.0 and 7.0.[22] In our study, the pH values for NE and NEG formulations range from 4.63 to 5.73 (Table 3). The difference between the pH values of B-NE and Hp-Et-NE formulations was significant (P<.05). Similarly, the difference between the pH values of B-NEG and Hp-Et-NEG formulations was also statistically significant (P<.05). It was observed that a slight decrease in the pH of the formulations occurred in the presence of the extract (Table 3). The pH of Hp-Et-NE and Hp-Et-NEG formulations was within the acceptable range for topical application to the skin.

FT-IR spectra of Hp-Et, NE, and NEG formulations are presented in Figures 3a and 3b. A broad band (at around 3300 cm^{-1}) associated with O-H groups was observed in the FT-IR spectrum of Hp-Et. In addition, we observed C-H stretching vibration at 2923 cm^{-1}, C=C and C=O stretching vibrations at 1510 cm^{-1}-1735 cm^{-1}, C–O stretching vibrations at 1035-1246 cm^{-1} and C–H bending vibrations at 1455 cm^{-1} (Figures 3a and 3b). These results are consistent with the literature.[23]

The B-NE and Hp-Et-NE formulations and similarly the B-NEG and Hp-Et-NEG formulations have similar spectra. The peaks belonging to Hp-Et were not observed in the FT-IR spectra of Hp-Et-NE and Hp-Et-NEG.

Table 3. NE and NEG formulations' pH values (Mean±SD; n=3)

Formulation	pH
B-NE	4.84±0.02
Hp-Et-NE	4.63±0.01
B-NEG	5.73±0.01
Hp-Et-NEG	5.46±0.01

The rheograms of NE and NEG formulations are shown in Figures 4a and 4b. In addition, the viscosity and "n" (flow behavior index) values determined for these formulations are given in Table 4. Viscosity affects the features such as stability, skin feel and penetration, and spreadability.[24,25] In our study, the viscosity values of B-NE and Hp-Et-NE formulations were around 2 cP (P>.05, Table 4). The viscosity values of B-NEG and Hp-Et-NEG formulations were determined as 78.19 cP and 75.97 cP, respectively (P>.05, Table 4). In addition, the "n" values were around 1 for NE formulations (n=1 Newtonian behavior[26]) and less than 1 for NEG formulations (pseudoplastic behavior[26]).

Therefore, the NE-based gel formulations which was prepared have suitable rheological properties (pseudoplastic behavior) for topical application.

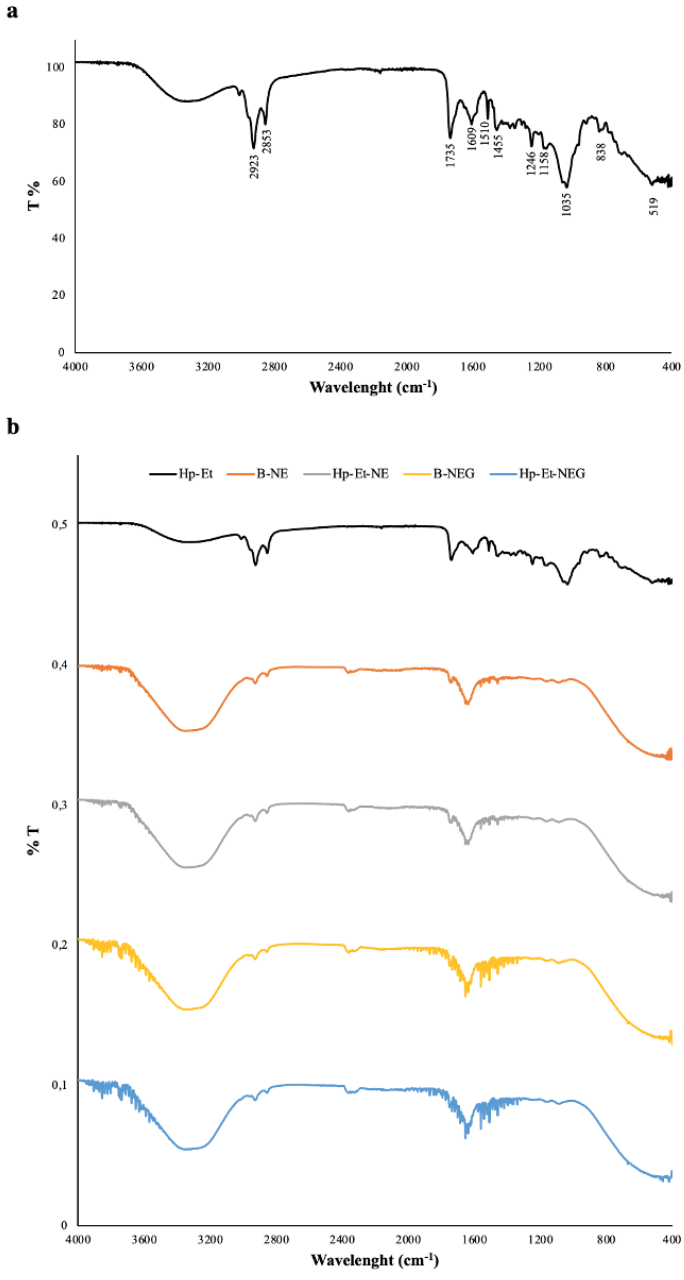

Figure 3. FT-IR spectra of Hp-Et (a and b), NE and NEG formulations (b)

Table 4. The viscosity and "n" (flow behavior index) values of the prepared formulations (Mean±SD; n=3)

Formulation	Shear rate (s^{-1})	Viscosity (cP)	n
B-NE	1500	2.14±0.05	1.012
Hp-Et-NE		2.20±0.01	1.043
B-NEG	300	78.19±1.59	0.865
Hp-Et-NEG		75.97±1.36	0.873

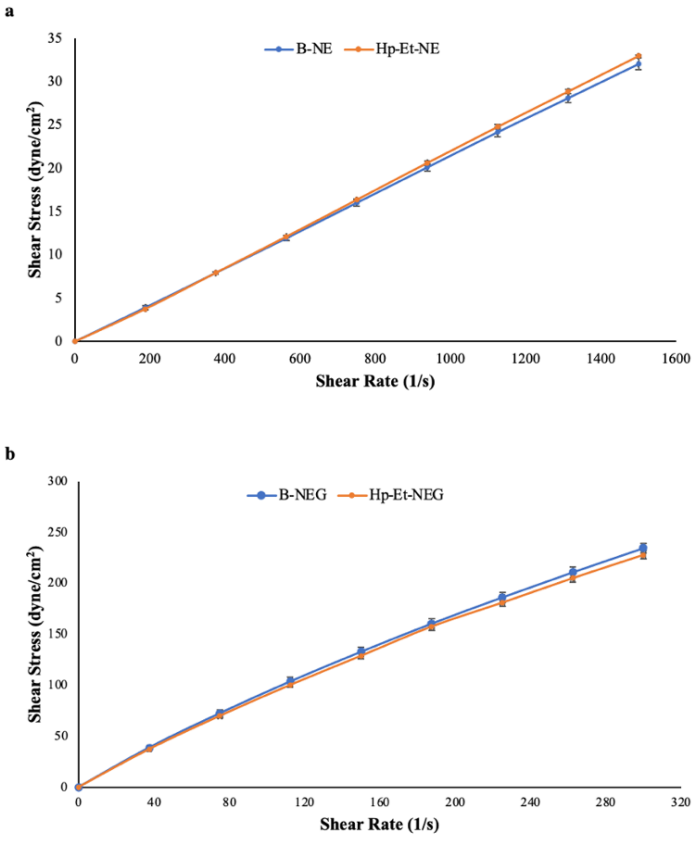

Figure 4. The rheograms of B-NE, Hp-Et-NE (a), B-NEG and Hp-Et-NEG (b) formulations (Mean±SD; n=3)

CONCLUSION

In our study, NE and NEG formulations containing Hp-Et were prepared for topical use for wound healing, and *in vitro* characterization studies were carried out. Hp-Et-NEG formulations had suitable pH and rheological properties (pseudoplastic behavior) for topical application to the skin.

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