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Araştırma Makalesi / Research Article

Doğal Fermente Portakal (*Citrus sinensis*) Kabuğu Sirkesinin Antibakteriyel ve Antioksidan Özelliklerinin Belirlenmesi / *Determination of Antibacterial and Antioxidant Properties of Naturally Fermented Orange (Citrus sinensis) Peel Vinegar*

Ruhşen AYDIN KARAAĞAÇ [1]; Mervenur GÜN[2]

1* Öğr.Gör.Dr., Kastamonu Üniversitesi İhsangazi Meslek Yüksekokulu, Veterinerlik Bölümü, raydin@kastamonu.edu.tr

2. Öğrenci, Kastamonu Üniversitesi, İhsangazi Meslek Yüksekokulu

Özet:

Bu çalışmada, geleneksel yöntemlerle üretilen portakal kabuklarından elde edilen sirkenin antioksidan ve antibakteriyel özellikleri araştırılmıştır. Portakal kabukları, fenolik bileşikler, flavonoidler ve C vitamini gibi yüksek miktarda biyolojik olarak aktif maddeler içerir. Bu bileşikler, mikroorganizmaların çoğalmasını engelleme ve serbest radikalleri nötralize etme potansiyeline sahiptir. Agar difüzyon yöntemi kullanılarak, portakal kabuğu sirkesinin test mikroorganizması Escherichia coli üzerindeki antibakteriyel etkisini araştırılmıştır. Sonuçlar, E. coli'nin portakal kabuğu sirkesine duyarlı olduğunu göstermiştir. Portakal kabuğu sirkesinin 2,2-difenil-1-pikrilhidrazil (DPPH) ve 2,2'-azinobis (ABTS) Radikal Temizleme Testi, Fe3+ ve Fe3+-TPTZ İndirgeme Kapasitesi Testi özellikleri belirlenmiştir. Portakal kabuğu sirkesi içerdiği C vitamini, doğal asitler ve biyoaktif bileşikleri sayesinde hem sindirime yardımcı olur hem de bağışıklığı destekler.

Bu çalışma ayrıca atık portakal kabuklarının biyoteknolojik değerlendirmesi için yenilikçi bir yaklaşım sunar ve çevresel sürdürülebilirliğe katkıda bulunur. Meyve kabuklarının bu şekilde kullanımı, atık bertarafıyla ilgili çevresel endişeleri ele alarak tarımsal yan ürünlerin geri dönüştürülmesine yönelik sürdürülebilir bir yaklaşım sunar.

Anahtar Kelimeler: Portakal kabuğu, sirke, Antibakteriyel Aktivite, Antioksidan Aktivite

Abstract:

In this study, the antioxidant and antibacterial properties of vinegar produced using traditional methods from orange peel was investigated. Orange peel contains high amounts of biologically active substances such as phenolic compounds, flavonoids, and vitamin C. These compounds

have the potential to inhibit the proliferation of microorganisms and neutralize free radicals. The agar diffusion method was used to investigate the antibacterial effect of orange peel vinegar on the test microorganism *Escherichia (E.) coli* . The results showed that *E. coli* was sensitive to orange peel vinegar. 2,2-difenil-1-pikrilhidrazil (DPPH) and 2,2'-azinobis (ABTS) Radical Scavenging Assay, Fe3+, and Fe3+-TPTZ Reducing Capacity Assay properties of orange peel vinegar were determined. Orange peel vinegar helps digestion and supports immunity thanks to the vitamin C, natural acids and bioactive compounds it contains. This study also presents an innovative approach for the biotechnological evaluation of waste orange peels and contributes to environmental sustainability. This use of fruit peels offers a sustainable approach to recycling agricultural by-products by addressing environmental concerns related to waste disposal.

Keywords: Orange peel, vinegar, Antibacterial Activity, Antioxidant Activity

1.Introduction

Citrus fruits, belonging to the Aurantoideae subfamily of the Rutaceae family, are the primary sources of vitamin C and have traditionally been used to treat various ailments. Although there are many species, the most important cultivated citrus fruits are *Citrus sinensis* (sweet orange), *Citrus reticulata* (mandarin orange), *Citrus limon* (lemon), *Citrus grandis* (pummelo), and *Citrus paradisi* (grapefruit) (Yılmaz, 2002). According to statistics, sweet oranges account for approximately 60% of fresh fruit and processed juice production among citrus fruits. In addition, citrus fruits and their juices are the most important sources of vitamin C, an important component of human nutrition (Xu *et al.,* 2012). It has also been traditionally used to treat many ailments such as constipation, cramps, colic, diarrhea, bronchitis, tuberculosis, cough, cold, obesity, menstrual disorders, angina, hypertension, anxiety, depression, and stress (Favela-Hernández *et al.,* 2016). These positive effects on human health have significantly increased the consumption of citrus fruits in recent years (Kamran Khan *et al.,* 2010). Most of the antioxidants found in plants, phenolics with biological effects such as antibacterial, antiviral, anti-inflammatory, anti-allergic, and antithrombotic, have been reported in studies to be present not only in the edible parts of the plant but also in the inedible parts of the plants (Rafiq *et al.,* 2018). By-products obtained from citrus waste are considered an economical and renewable source of valuable compounds that can be used in the pharmaceutical, nutraceutical, food, and cosmetic industries (Mahato *et al.,* 2018). Citrus fruits are widely consumed fresh and as juice worldwide, and their peels, which have significant antioxidant activity and a wide variety of secondary components compared to other parts of the fruit, are usually processed as by-products or discarded, causing environmental pollution (Londono *et al.,* 2010). Today, citrus waste disposal strategies (burning or landfilling) are inadequate and problematic regarding environmental impacts and energy efficiency (Satari and Karimi, 2018). Their disposal requires high costs, and their unprocedural disposal can harm living organisms in the soil microbiota due to the toxicity of the volatile oils in citrus fruits. Fruit wastes are rich in bioactive compounds and have recyclable properties as low-cost, readily available, value-added food supplements. *Escherichia (E.) coli* is a Gram negative (-) bacteria. Although some species of *E. coli* are not harmful to animals, they can be a cause of illness when transmitted to humans. The main diseases it causes are diarrheal, urinary tract infections, meningitis, peritonitis, mastitis, septicemia, and pneumonia. It has been shown that *E. coli* can cause disease in animals other than humans, such as chickens and calves (Bilgehan, 2000). In any sample, *E. coli* in the intestinal systems of warm-blooded animals (mammals and poultry) indicates direct or indirect fecal contamination (Çakır, 2000). It is known that vinegar has

antimicrobial properties that are used to clean and treat nail fungus, head lice, warts, and ear infections (Rutala *et al.*, 2000; Dohar, 2003). Consumers prefer natural preservative methods to inhibit the growth of foodborne pathogenic microorganisms that may occur in foods (Rauha *et al.*, 2000).

Nowadays, vinegar produced by adding artificial acids has an intense odor and a sharp taste and is produced quickly. Due to the disadvantages, such as excessive use of antimicrobial agents, microbial resistance, and imbalance in oral flora, alternative new antimicrobial agents that are safe and specific for oral pathogens have begun to be investigated. Using plants as therapeutic agents attracts attention due to their low negative effects. In some Western countries, such as America and Canada, many vinegars, such as apple cider vinegar, are consumed by mixing with fruit juice to treat some diseases (Giudici *et al.*, 2015). Oxygen (O2) can be toxic to aerobics at very high concentrations. Reactive oxygen species (ROS) containing oxygen can be very dangerous due to unpaired electrons. This can cause harmful effects on cell structures. Antioxidant compounds absorbed from our diet are thought to play a role in preventing cancer, cardiovascular diseases, and other age-related diseases. Due to concerns about the safety of some synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) , widely used in processed foods, the demand for healthier and more natural antioxidants has increased (Anagnostopoulou, 2006; Rafiq *et al.*, 2018). Citrus waste by-products, including peels, provide a sustainable and economical source of valuable compounds for the pharmaceutical, food, and cosmetic industries. Vinegar, traditionally produced by alcohol and acetic acid fermentation, is known for its antimicrobial properties and natural preservative potential.

2. Material and Methods

2.1.Obtaining Vinegar from Orange Peel

Vinegar production consists of a two-stage fermentation process: alcohol fermentation and acetic acid fermentation. The first stage is alcohol fermentation, which is the conversion of fermentable sugars into ethanol and carbon dioxide by yeasts of the *Saccharomyces* species under anaerobic conditions. The second stage is acetic acid fermentation, in which the alcohol formed in the first stage is converted into acetic acid and water under aerobic conditions by acetic acid bacteria such as *Acetobacter aceti, Acetobacter pastorianus* and *Acetobacter hansenii* (Plessi, 2003). The fact that the membrane formed by acetic acid bacteria on the surface of the liquid has settled to the bottom and its pH is around 3.83 may also give an idea that vinegarization is complete. Vinegar production is completed with this method (Özkaya *et al.*, 1991; Aktan & Kalkan, 1998; Plessi, 2003). In our study, vinegar was established from orange peel in accordance with the procedure mentioned (slow method).

2.2.Antibacterial Activity Test

Kirby and Bauer's disk diffusion susceptibility test (DDDT) was used to determine the antimicrobial activities of vinegar and its dilutions. The previously prepared orange peel vinegar was passed through a membrane filter (0.2 µm) and sterilized, and 400 µl amounts were impregnated onto 6 mm diameter blank sterile disks (OXOID susceptibility Blank disk) (1mg/disc). The disks were used after drying at 37ºC. OFX=Ofloxacin (10Tg/disc) or SCF=sulbactam (30Tg) + cefoperazone (75Tg) (105Tg/disc) standard antibiotic disks were used as

a positive control, and pure water was used as a negative control. Colonies from 18 24 hour fresh cultures of *E. coli* produced on solid media (plate culture) were suspended in physiological water and then cultured on the surface of petri dishes containing Nutrient Agar (NA). Then, the disks were placed on the petri dishes appropriately and incubated at 37ºC for 24 hours. The diameters of the inhibition zones formed at the end of incubation were measured with a millimeter ruler. Each assay in this experiment was repeated 3 times and determined the arithmetical average of the result. *E. coli* was provided from Kastamonu Training and Research Hospital (Kastamonu, Türkiye), used as a test microorganism.

2.3.Measurement of Antioxidant Activity

2.3.1.DPPH and ABTS radical scavenging assay

The DPPH radical scavenging activity determination assay was performed as previously applied (Karagecili *et al.,* 2023; Karagecili et al., 2023*). An ethanol solution of 0.1 mM DPPH was prepared and incubated in the dark by mixing overnight for preradicalization. Then, 0.5 mL of DPPH and 0.5 mL of the samples in ethanol (15-45 µg/mL) were mixed and incubated at 30 ºC for 30 minutes. The absorbances of each sample were recorded at 517 nm. ABTS radical scavenging was performed based on the bleaching ability of different concentrations of the extracts (Karagecili *et al.*,2023; Gulcin,2020). Preradicalized ABTS radicals were obtained by mixing an equal volume of 2.45 mM potassium-thiosulfate and 2 mM ABTS for 6 hours in the dark. Then, the absorbance of ABTS radical solution at 734 nm was maintained at approximately 1.0. The assay was performed by incubating ABTS radicals and 3 mL of the samples (15-45 µg/mL) in 0.1 M PBS (pH=7.4) for 30 minutes. The bleaching ability of the samples was measured by the decrease in absorbance at 734 nm. DPPH and ABTS assays were performed with ascorbic acid, BHA, BHT, and Trolox. Each sample was performed in triplicate.

2.3.2.Fe3+ and Fe3+-TPTZ reducing capacity assay

The ability of the samples to reduce metal complexes, in other words, the capability to donate one electron to an acceptor, was investigated with three different methods conducted in triplicate for each sample and compared with the positive controls, BHA, BHT, and Trolox.

1)Fe3+ reducing assay; An amount of 0.75 mL of three different concentrations of the samples in distilled water were mixed with 1.25 mL of 0.20 M phosphate buffer solution (pH 6.6) and 1% (w/w) potassium ferrocyanide. Then, the mixture was incubated at 50 ºC for 30 min before both acidifying media with 1.25 mL of 10 % trichloroacetic acid (w/w), were used to form Perl's Prussian blue complex, which gives absorption maxima at 700 nm with the addition of 0.25 mL of 0.1 % iron(III) chloride (Karagecili *et al.,* 2023; Ozden *et al.*,2023). Spectral color changes of yellow solution into green or blue color, depending upon reducing the capacity of the samples, were observed, and reaction mixtures were analyzed against a sample that contained distilled water instead of sample solution via Shimadzu® UV-1800 UV Spectrophotometer (Shimadzu Corporation/Japan).

2) Fe3+- TPTZ reducing assay; In acidic environments, Fe3+-(TPTZ) 2 complexes take one electron from a reducing agent, transform into Fe+2-(TPTZ) 2 complex, therefore intensive blue color takes place in the reaction mixture (Karagecili *et al.,* 2023; Karagecili *et al.,* 2023*). demir (III) iyonu indirgeyici antioksidan güç yöntemi (FRAP) reagent containing 10 mM TPTZ (in 0.4 mM

hydrochloric acid): 20 mM iron(III) chloride: 0.3 M sodium acetate buffer (pH=3.6) in a ratio 1:1:10 was prepared before use. A total of 0.5 mL of the samples in acetate buffer was mixed with an equal volume of 20 mM iron (III) chloride and FRAP reagent, resulting in a final reaction volume of 5 mL. The sample containing buffer instead of the samples was used as blank, and the absorbance of each reaction was measured at a wavelength of 593 nm after 30 min incubation at 37 ºC.

3. Results & Discussion

3.1.Antibacterial activity of Orange Peel Vinegar

The zone diameters formed according to the results of Kirby and Bauer's disk diffusion susceptibility test (DDDT) used to determine the antimicrobial activity of vinegar are shown in Table 1. *Escherichia coli* was determined to be sensitive to orange peel vinegar. While ofloxacin and sulbactam + cefoperazone, which we used as positive controls, were seen to be effective on the test microorganism, it was seen that the negative control prepared by impregnation of only pure water did not form an inhibition zone.

Table 1. Diameter of inhibition zone (mm)

Test microorganism	Orange peel vinegar	Negative control pure water	Positive control OFX=Ofloxacin (10Tg/disc)
Escherichia coli	6mm	-	25mm

According to the results of this research, it has been seen that the antibacterial effect of the orange peel vinegar on *E. coli*. However, it would be seen normally the emergence of different results if we evaluated the changing conditions, such as the geographical region where the orange tree is grown, used bacterial strains, and the working medium.

3.2. Measurement of Antioxidant activity of Orange Peel Vinegar

3.2.1.Radical scavenging activity assays

Results obtained from the DPPH radical scavenging assay showed that orange peel vinegar has a greater DPPH radical scavenging activity with a 16.19 ±1.52 IC50 value. It has a scavenging ratio of up to 88.46 % when it is applied at a concentration of 45 µg/mL. When the performance of the sample was compared to the standards as follows in descending order: Trolox> ascorbic acid>BHT>BHA>Sample-1 (12.99± 1.81>14.28± 1.63>15.07± 0.98>16.00± 1.58>16.19± 1.52). The sample showed DPPH radical scavenging results that were close to those of synthetic and natural analogs (Figure 1).

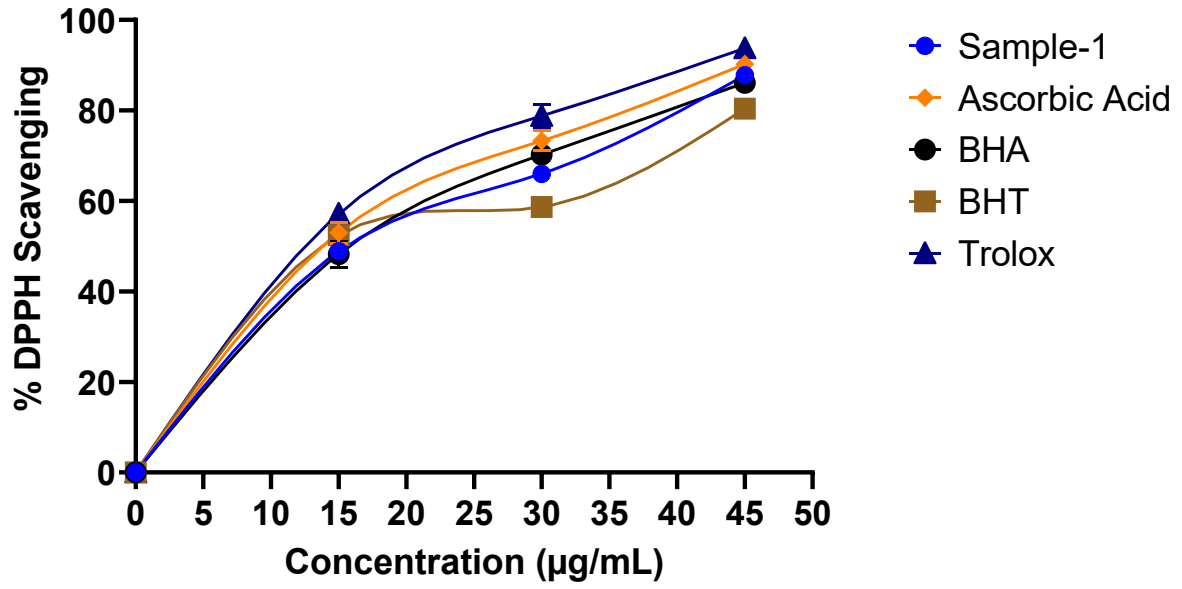

Figure 1. DPPH results of the vinegar sample.

The ABTS radical scavenging properties of the sample showed similar results to those obtained from the DPPH assay. The sample yielded better ABTS radical scavenging properties with a 9.30±1.98 IC50 value. Radicals of ABTS were scavenged by 45 µg/mL of sample 1 up to 97.92% (Figure 2). The sample and standards were scavenged from the ABTS radical in descending order as follows:

Trolox>BHA>Sample-1>BHT (6.30 ±1.71>8.46 ±1.92>9.30± 1.98>13.79± 2.68).

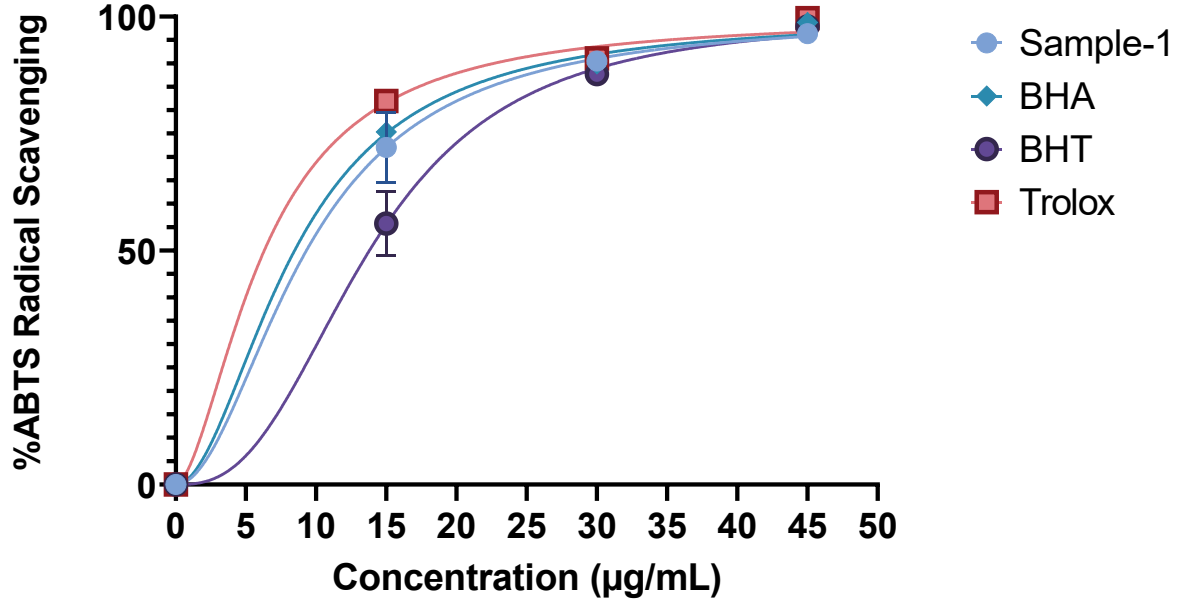

Figure 2. ABTS results of the vinegar sample.

3.2.2.Metal reducing assays

Fe3+-reducing and Fe-TPTZ complex-reducing activities were investigated for the sample, and quite different results were obtained from the two assays. Although the Fe-TPTZ reduction capacity of the sample was lower than the standards, the remarkable metal reduction was recorded at the dose of 45 µg/mL (Figure 3): BHT>Trolox>BHA>Sample-1 (2.87± 0.06>2.48± 0.18>2.33± 0.01>1.18± 0.10).

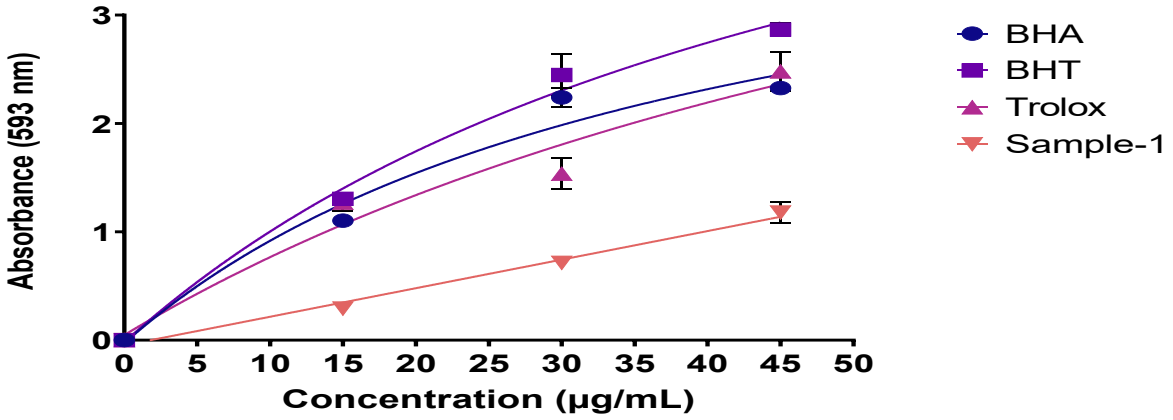

Figure 3. Fe-TPTZ reducing results of the vinegar sample.

According to the Fe3+-reducing results, the sample showed a remarkable capacity to reduce Fe3+ as much as the synthetic analog, Trolox. Fe3+ reducing capacities of the samples and standard antioxidants in descending order were as follows (Figure 4):

BHA>BHT>Sample-1>Trolox (1.75± 0.04>1.50± 0.08>1.34± 0.03>0.84± 0.01).

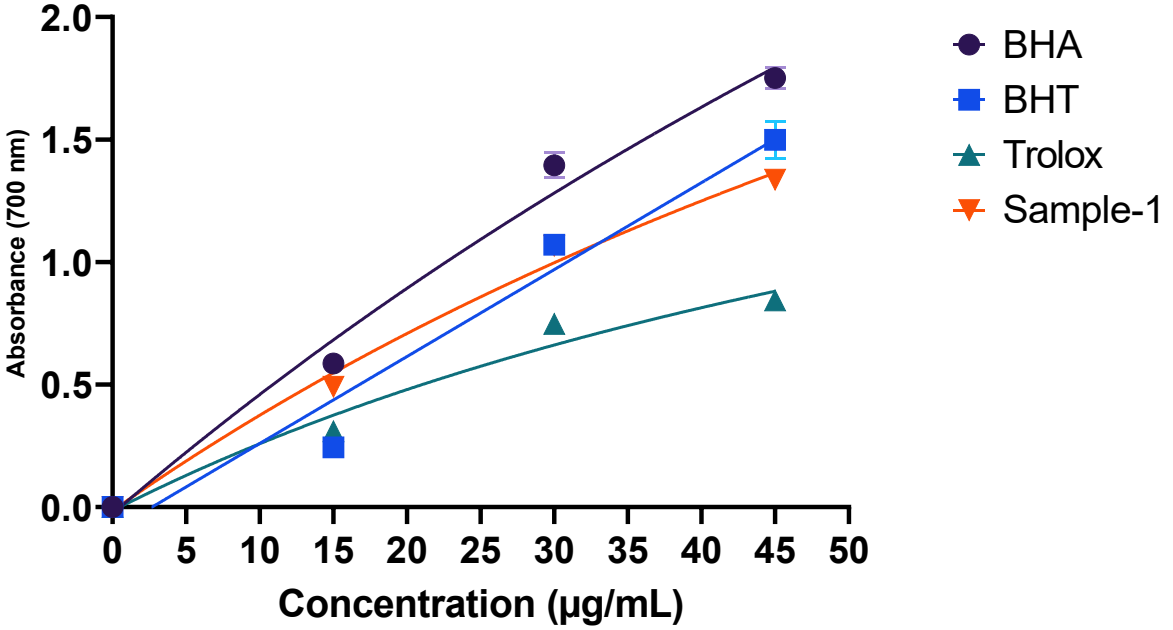

Figure 4. Fe3+-reducing results of the vinegar sample.

Table 2. Antioxidant activity findings of orange peel vinegar

Method	DPPH radical scavenging assay (IC_{50})	ABTS radical scavenging assay (IC_{50})	FRAP (λ_{593})	Fe^{3+} (λ_{700})
BHA (45 µg/mL)	16.00±1.58, r^2=0,99	8.46 ±1.92, r^2=0,99	2.33± 0.0, r^2=0,96	1.75± 0.04, r^2=0,99
BHT (45 µg/mL)	15.07±0.98, r^2=0,97	13.79± 2.68, r^2=0,99	2.87± 0.06, r^2=0,98	1.50± 0.08, r^2=0,96
Trolox (45 µg/mL)	12.99±1.81, r^2=0,99	6.30 ±1.71, r^2=0,99	2.48± 0.18, r^2=0,94	0.84± 0.0, r^2=0,97
Ascorbic Acid(45 µg/mL)	14.28±1.63, r^2=0,99	-	-	-
Sample-1 (45 µg/mL)	16.19±1.52, r^2=0,98	9.30± 1.98, r^2=0,99	1.18± 0.10 r^2=0,98	1.34± 0.03, r^2=0,99

The findings of the study on the antibacterial and antioxidant activity of orange peel vinegar underscore its potential as a natural, cost-effective alternative to synthetic preservatives and antimicrobial agents. The use of orange peels, often discarded as waste, transforms a significant environmental pollutant into a valuable resource, aligning with sustainable waste management practices and the circular economy. The significant antimicrobial activity observed highlights orange peel vinegar's potential application in food preservation and as a natural disinfectant. Its ability to inhibit the growth of foodborne pathogens offers an alternative to synthetic antimicrobial agents, which are associated with microbial resistance and potential health risks. This is particularly valuable for consumers seeking safer, natural methods to reduce spoilage and contamination in food.

The study also points out that the antioxidant properties of orange peel vinegar are not limited to the edible portion but extend to the inedible peel, maximizing the use of citrus fruits. This adds value to a typically discarded by-product, creating opportunities for its use in nutraceutical and cosmetic industries.

Declarations:

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