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Enhancing the Antibacterial Effect by Adding MWCNT and ZrO2 Nanomaterials to Extracts Obtained from Different Parts of Persea americana Fruit

Avokado Meyve Özlerinin Antibakteriyel Etkisinin MWCNT ve ZrO2 Nanomalzemeler Eklenerek Artırılması

Yeşim Dağlıoğlu[1]
Ömer Ertürk[2]

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ABSTRACT:
In this study, Avocado fruit (Persea americana) that reached eating maturity was used. Extracts of dissimilar dehydrated parts of exocarp, pericarp and seed (avocado fruits) with ethanol were acquired. MWCNT and ZrO_2 nanomaterials were added to these extracts obtained from different parts of avocado. Then, the antibacterial activity of these extracts was tested by disk diffusion test using *Staphylococcus aureus* and *Bacillus subtilis* (gram+), *Escherichia coli* (gram-) bacteria. Antibacterial activity was evaluated by measuring the zone diameter formed after 24 hours of incubation. MWCNT and ZrO_2 indicated a synergistic effect with avocado fruit extracts and significantly increased the antibacterial activity of extracts of dissimilar parts of avocado (*P<0.05*)

Keywords: Antibacterial activity, avocado, MWCNT, *zirconium oxide*, *Persea americana*

ÖZ:
Bu çalışmada yeme olgunluğuna ulaşmış Avokado (*Persea americana*) meyvesi kullanılmıştır. Etanol ile ekzokarp, perikarp ve tohumun (avokado meyveleri) farklı kurutulmuş kısımlarının özleri elde edilmiştir. Avokadonun farklı kısımlarından elde edilen bu ekstraktlara MWCNT ve ZrO_2 nanomateryalleri eklenmiştir. Daha sonra bu ekstraktların antibakteriyel aktivitesi, *Staphylococcus aureus* ve *Bacillus subtilis* (gram+), *Escherichia coli* (gram-) bakterileri kullanılarak disk difüzyon testi ile test edildi. Antibakteriyel aktivite, 24 saatlik inkübasyondan sonra oluşan zon çapı ölçülerek değerlendirildi. MWCNT ve ZrO_2, avokado meyve özleri ile sinerjistik bir etki göstermiştir ve avokadonun farklı kısımlarından elde edilen özlerinin antibakteriyel aktivitesini önemli ölçüde artırmıştır (P<0.05).

Anahtar Kelimeler: Antibakteriyel aktivite, avokado, MWCNT, zirkonyum oksit, *Persea americana*

1.GİRİŞ:

Mankind has always seen nature as a natural medicine store since its existence. The place of medicinal plants in history dates back to 5000-3000 BC. Mankind has used medicinal and aromatic plants collected from nature for a wide variety of purposes for centuries. For example, medicine, spice, pharmaceutical industry, beverage, soap, perfume, cosmetics, confectionery, chewing gum, toothpaste, relaxing tea and medicinal manufacturing, aroma, essence, ornament, landscape and many other fields. Today, its most important use is as a pharmaceutical raw material. Medicinal and aromatic plants have been taken into account by modern medicine as an alternative to reduce the harmful side effects of drugs that are frequently used today (Mindell, 2003). However, with the rapidly growing world population, the demand for food is also increasing (WHO, 2022). This has led to the reality of access to food that is safe for human consumption. Bacterial contamination is the biggest threat to food safety (Conte, 2007). Synthetic additives are thoroughly used in the food industry to control microbial pathogens and to prevent microbial decay. However, in recent years, the demand for natural food additives and plant extracts has increased (Conte et al., 2007).

1 **Corresponding Author:** OrduÜniversitesi, yozkan52@gmail.com, ORCID: 0000-0001-8740-1162
2 Ordu Üniversitesi, oseerturk@hotmail.com, ORCID: 0000-0001-5837-6893

One of these plant extracts is Persea americana (Mill.), also known as avocado. *P. americana* first appeared in the Tehuacan Valley of Mexico (Landon, 2009). Avocado, which belongs to the family of Lauraceae, contains various potential vitamins, minerals. It contains carotenoid antioxidants such as glutathione, lutein, astaxanthin, zeaxanthin, and violaxanthin, as well as phytochemicals with numerous potential health utility. It also contains compounds found in the lipid fraction, such as squalene. Of these, lutein is an antioxidant that protects the eye against macular degeneration and cataract formation. It has also been suggested that lutein positively affects vascular diseases and diabetic retinopathy by modulating reactive oxygen species (ROS) fabrication. Thus, it also has an important role in preventing diseases by slowing the aging process (Lidebjer et al., 2007). At the same time, avocado fruit has antioxidant, hypoglycemic, antihypertensive, antiobesity, anticarcinogenic, hypolipidemic, anti-inflammatory, anticonvulsant, anti lithiasis, antiosteoarthritis, antitumor, antimicrobial, antiprotozoal, antimycobacterial, hepato and chemoprotective activities (Daiuto et al., 2010; Tabeshpour et al., 2017). Many studies have been conducted on avocado's prevention of cardiovascular diseases (Grant, 1960). Its high potassium content helps against cardiovascular diseases and regulates muscle activity. The antioxidant glutathione source and carcinogens are highly effective on compounds (Duarte et al., 2016). Its β-sitosterol content is used against infections by strengthening the immune system and in treating diseases where immunity is reduced, such as cancer and HIV. However, this compound also increases lymphocyte proliferation and activity of natural killer cells, which inactivates invading microorganisms (Bouic, 2002). β-sitosterol activity also helps weight loss by reducing body fat accumulation (Duarte et al., 2016). Phytochemicals such as isorhamnetin, luteolin, rutin, quercetin, and apigenin isolated from avocado leaves can also help prevent the progression of various diseases against oxidative stress (Owolabi et al., 2010). In particular, extracts from the epicarp of the unripe avocado fruit have been shown in previous studies to have both antifungal and antibacterial properties (Jacob et al., 1971; Sivanathan & Adikaram,1989). The seed of the immature fruit also has antibacterial properties (Jacob et al., 1971). Tannins, catechin flavones, and polyphenolic compounds are commonly found in the tissues and seeds of the *P. americana* fruit. These chemicals are antimicrobial in nature and contribute to the antibacterial activity of the unripe fruit (Jacob et al., 1971; Young & Biale 1967). In short, the most abundant bioactive components in *P. Americana* are carotenoids, fatty acids, minerals, phenolic and polyphenolic compounds, phytostanols and phytosterols, seven-carbon sugars, proteins, vitamins (Tabeshpour et al., 2017). These bioactive compounds of *P. americana* provide its antimicrobial activity.

The rapid development of nanotechnology and nanoscience in recent decades has allowed nanomaterials to be used as an alternative in antibacterial therapy. The properties of nanomaterials, such as increased membrane permeability, efflux pump inhibitors, and potential multiple antibacterial effects, prevent the formation of bacterial resistance compared to conventional antibiotics (Huh & Kwon, 2011; Hajipour et al., 2012). In this study, the antibacterial effect of avocado fruit extracts as a result of interaction with multi-walled carbon nanotubes (MWCNTs) was investigated because CNTs can break the stages resistance of diverse bacterial strains (Yuan et al., 2008; Das et al., 2011; MubarakAli et al., 2011). In addition, CNTs are cheaper, less dispersive, and used in different functions than other antibacterial nanomaterials (e.g., silver nanoparticles), and they also have disinfection potential (Bianco, et al., 2005; Zhang & Oh, 2010; Zhou & Qi, 2011; Dağlıoğlu and Öztürk 2019). Despite the toxicity of CNTs, their functionalized products are considered much less toxic alternatives both *in vitro* and *in vivo* (Sayes et al., 2006; Kang et al., 2009; Arsawang et al., 2011). Zirconium (Zr) is a multifunctional element with a wide range of applications thanks to its superior photochemical properties such as mechanical, electrical, thermal, and optical (Sigwadi et al., 2019). It is used as a catalyst in potential biomedical usage in medicine and dentistry for therapeutic purposes (Hu et al., 2019). In addition, anticancer, antioxidant, and antibacterial properties of ZrO_2 nanoparticles have been reported (Mftah et al., 2015; Balaji et al., 2017).

The purpose of this study is to investigate the comparative antimicrobial effect of ethanol extracts prepared from different parts of the widely consumed *Persea americana* (avocado) fruit, such as pericarp, exocarp, and seed, and the nanomaterial mixtures and nanomaterials added to these extracts. This is a pioneering study because it is the first scientific record that the antibacterial effect of extracts from different parts of the avocado fruit is enhanced by interaction with nanomaterials.

2.MATERIAL and METHOD

2.1.Preparation of avocado extracts

Avocado fruit, which has reached maturity, was obtained from the gross market in Ordu/Turkey province in 2018. After about 200 g of the pericarp, seed, and exocarp parts of the avocado fruit were dehydrated and powdered, the extract was prepared according to Holopainen. The extracts prepared by making small changes in this method were dried at 20-22 ºC and in the dark (Holopainen et al., 1998; Dağlıoğlu and Öztürk 2016; Dağlıoğlu et al., 2016a,b). After dehydration, it was pulverized with a blender. This dust sample was then extracted at a ratio of 1 to 5 in 95% ethanol at 20-22 ºC.

The obtained extracts were stored in dark glass bottles at +4 ºC for 3 days to ensure homogenization. Just before the experiment, the extracts were filtered first through a rude filter and then entirely a 45 µm membrane filter. In the next step, ethanol was taken out from the avocado extracts with the help of an evaporator and dehydrated. The dust extracts were hidden in the refrigerator at -20 ºC until in antibacterial activity assay.

2.2.Preparation of nanomaterials

MWCNT (Multi-walled carbon nanotube) which is 90% pure with an average size of 5-10 nm, ZrO_2 (zirconium oxide) has an average size of 40 nm and a purity of 99.5% and was provided from Ankara/Turkey Nanography. Extracts of different parts of avocado were suspended with MWCNT and ZrO_2 in ddH_2O. This suspension was homogenized by sonicating for 30 minutes with a sonicator. Wide-range dose-response studies were performed to determine assay concentrations of antibacterial. The optimal concentration range was found to be 1-48 mg/L for ZrO_2 and 5-80 mg/L for MWCNT.In these positioning studies, optimal options of 10 mg/L for MWCNT and 4 mg/L for ZrO2 were selected. Then, avocado extracts that we prepared at the same rate were added to these concentrations; that is, the nanomaterial extract ratio is 1:1. Characterization of nanomaterials was analyzed using a by scanning electron microscope (Hitachi SU-510, japan).

2.3.Disk Diffusion method

In this study, the disc diffusion method was chosen to determine the antimicrobial activity. Antimicrobial activity was determined using three different pathogens: gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and gram-negative (*Escherichia coli*). Mueller Hinton Agar (Oxoid, CM0337) medium was used in the disc diffusion method to investigate the antibacterial activity of the extract-nanomaterial mixture. Activated bacteria were set to McFarland 0.5 value and after inoculation into the medium, the discs prepared with 100 µg/mL nanomaterial (1:1) in 100 µg/mL extract were placed in the medium. Finally the 24 h incubation period at 37°C, the inhibition zone diameters formed in the medium were measured in mm. Experiments were repeated three times (Dağlıoğlu et al., 2023).

2.4.Statistical analysis

The antibacterial assays were duplicated four times and the arithmetic average of the four data acquired was received. Zone diameter data were applied one-way analysis of variance (ANOVA) to detect the significance of single differences at a significance level of $p < 0.05$.

3. RESULTS AND DISCUSSION

The World Health Organization (WHO) warned about antibiotic resistance and declared that none of the 43 antibiotics under development in 2021 were sufficient to combat antibiotic resistance (Chen et al., 2008). Due to antibiotic resistance, thousands of people die each year in the ABD from infectious bacterial diseases such as pneumonia and dysentery (Aslan et al., 2010). In addition, the number of deaths from infectious diseases is increasing worldwide (Cohen, 2000). The most commonly used antibiotics in the treatment of bacterial infectious diseases are mainly penicillin, kanamycin and spectinomycin. Whereas, bacteria resistance happens when these antibiotics are used unconsciously and unnecessarily (Amyes & Gemmell, 1992). Now then, it is required to improve new antibacterial agents in the treatment of these diseases. Previous records mentioned the potential uses of the uneatable pieces of avocado fruit as cheap bioactive compounds for personal care products, nutrients, and medicines (Vinha et al., 2012; Dağlıoğlu et al., 2022).

In this study, avocado fruit, which is known to have antibacterial properties due to many bioactive compounds in its content, was selected. The synergistic effect of this fruit with nanoparticles has been ensured to further increase its antibacterial activity.

Previous studies have joined with metallic nanoparticles besides biochemical features to improve the antibacterial activity of CNTs (Pantarotto et al., 2003; Mohan et al., 2011 ; Murugan et al., 2011). In this study, the antibacterial effect of avocado, which is known to have high antibacterial properties, was increased with MWCNT and ZrO_2, which are also known to have antibacterial properties. In Figure 1, it is seen that MWCNT and ZrO_2 NPs exhibit a one-dimensional morphological structure and are densely packed.

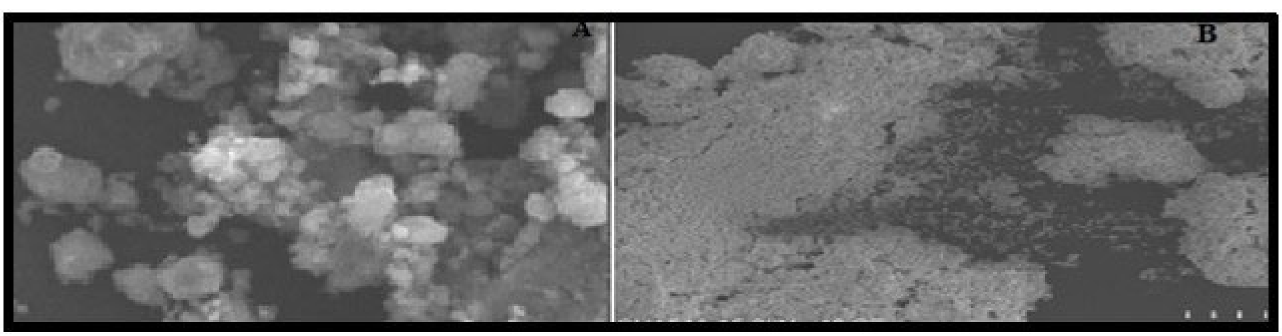

Figure 1. Scanning electron microscopy (SEM) micrographs of A) MWCNT, B) ZrO_2 NPs

The results of the antibacterial activity study are presented in Table 1. According to these data, it was noted that avocado fruit has a significant antibacterial effect from the seed and exocarp parts of the pericarp ($P<0.05$), In addition, MWCNT and ZrO_2 NPs were found to have much more antibacterial effects in avocado fruit parts. As a result of the interaction of avocado fruit parts with nanomaterials, it indicated a much higher antibacterial effect than both avocado fruit parts and nanomaterials. In particular, ZrO_2

NPs+seed, ZrO_2 NPs+exocarp and MWCNT+seed mixtures demonstrated the highest effect in gram-positive. In gram-negative bacteria, MWCNT+seed and ZrO_2 NPs indicated exocarp. When we evaluated gram-positive and negative, the nanomaterial and extract mixture showed antibacterial activity at a significant level (P<0.05).

Table 1. *Antibacterial activities of the extracts obtained from different parts of P. Americana, mixture with nanomaterials added to these extracts and nanomaterials against bacteria (disc diffusion test, 25 µg/mL)[a].*

Sample			Disc diffusion zone diameter (mm) with 20 µg disc		
			Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*
Seed			11.30 ± 0.33^{a}	14.65 ± 0.28^{b}	23.55 ± 0.14^{a}
pericarp			4.20 ± 0.12^{b}	4.10 ± 0.31^{c}	7.95 ± 0.18^{b}
exocarp			12.38 ± 0.51^{a}	$19.82^{*} \pm 0.37^{a}$	23.76 ± 0.22^{a}
MWCNT	+	seed	$19.85^{*} \pm 0.29^{a}$	$22.80^{*} \pm 0.21^{a}$	$31.50^{**} \pm 0.41^{a}$
MWCNT	+	pericarp	11.25 ± 0.34^{b}	11.15 ± 0.20^{b}	$17.10^{*} \pm 0.38^{c}$
MWCNT	+	exocarp	19.30 ± 0.33^{a}	$21.10^{*} \pm 0.30^{a}$	26.00 ± 0.39^{b}
ZrO_2 NPs	+	seed	$24.40^{*} \pm 0.12^{a}$	$24.70^{*} \pm 0.27^{a}$	$29.90^{*} \pm 0.21^{a}$
ZrO_2 NPs	+	pericarp	18.25 ± 0.10^{b}	19.50 ± 0.14^{b}	14.90 ± 0.25^{b}
ZrO_2 NPs	+	exocarp	18.30 ± 0.45^{b}	23.75 ± 0.41^{a}	$30.80^{**} \pm 0.25^{a}$
MWCNT			11.88 ± 0.25^{b}	15.25 ± 0.26^{b}	8.65 ± 0.18^{b}
Zr NPs			$17.00 \pm .0.15^{a}$	21.25 ± 0.00^{a}	13.78 ± 0.17^{a}
Gentamicin 10 (µg disc)			17.55 ± 0.10	19.48 ± 0.18	20.84 ± 0.02

[a]Data are means ± standard deviations of three single avocado extract, avocado extract+NPs and NPs for each variety. Different lowercase letters in the same column correspond to significant variation of the same bacterial strain for different cultivars by Tukey's test ($p < 0.05$).

Many studies have been conducted on the antibacterial effects of avocados before. While most studies so far have emphasized the antibacterial activity of extracts from varied avocado species, only a few have reported the opposite. One of these studies reported that avocado seed or avocado peel extracts did not show an antimicrobial effect against any of the bacteria examined (Calderón-Oliver et al., 2016). In another study, the antibacterial capacity of extracts with different polarities obtained from avocado seeds (*Persea americana* Mill cv. Lorena) was evaluated. As a result, she noted that none of the extracts indicated antimicrobial effect against *Escherichia coli* and *Staphylococcus aureus* (Rodriguez-Carpena et al., 2011). Researches have shown that avocado extracts have different antibacterial effects. The reasons for this include the different types of avocados, the variety of parts to be extracted (i.e. seed, exocarp, endocarp or mesocarp), the way the extraction is done (e.g. the type of solvent used), the maturity level of the avocado, and the bacterial species studied (Rodriguez-Carpena et al., 2011; Idris et al., 2009; Chia & Dykes, 2010; Amado et al., 2019). One study reported the antibacterial and antifungal features of extracts from the unripe avocado epicarp (Jacob et al., 1971; Sivanathan & Adikaram, 1989). Chia and Dykes 2010, in their study seeking the antibacterial effect of seed extracts of epicarp and three types of mature avocado fruit, indicated that crude extracts of epicarp and mature avocado seeds have antibacterial activity (Chia & Dykes, 2010; Shaw et al., 2020). In a previous study, the antibacterial effect of *P. amerciana* seed extracts against a large number of microorganisms was evaluated. The antibacterial effect of avocado extracted with chloroform, methanol and ethyl acetate by disk diffusion method has been demonstrated (Idris, et al., 2009).

In a recent study, the antioxidant and antibacterial properties of the peel, pulp and seed parts of four types of avocados, Quintal, Fortuna, Margarida and Hass, were investigated. They noted that the most sensitive microorganism to the extracts studied was *S. aureus* which demonstrated bacteriostatic and bactericidal effects, and the extract that gave the best results was the bark of the Quintal variety. In addition, extracts made from the pulp, the edible part of the avocado, reported almost no activity against the bacteria tested (Amado et al., 2019). Rodriguez-Carpena et al., 2011 evaluated the antibacterial activity of extracts from different parts (peel, seed and pulp) of a number of avocado cultivars against a range of bacteria and found that gram-negative bacteria demonstrated advanced inhibitory effect against gram-positives. That is, gram-positive bacteria have reported that they are more susceptible. The researchers stated that all avocado parts have antimicrobial properties, with the pulp (mesocarp) showing the highest activity (Rodriguez-Carpena et al., 2011). There is a study similar to our study. They noted that avocado seed extracts enriched with acetogenin (acetogenins, a long unsaturated aliphatic chain fatty acid derivative) indicated high antimicrobial activity against *L. Monocytogenes* (Salinas-Salazar et al., 2017). In a study, ZnO nanoparticles produced in the size range of 10-40 nm in diameter and edible ZnO/chitosan/gum Arabic coating were used as antimicrobial additives. As a result, this edible coating both extended the shelf life of the avocado and ensured its high performance. Thus, the appearance and freshness of the avocado with the edible coating is extended for 7 days (Le et al., 2021). In a study evaluating the antibacterial activity of ZnO nanoparticles synthesized by the green method using Avocado Seed Extract, the antibacterial effect of ZnO nanoparticles on S. aureus and E. coli was proven (Saridewi et al., 2022). *Persea americana seed coat-mediated hydronium jarosite nanoparticles have been noted to be insensitive to both Gram-negative and Gram-positive bacteria* (Botha et al., 2023). In the study where the antimicrobial and antioxidant activity of copper nanoparticles synthesized using *Persea americana* seeds was evaluated; Avocado seed-mediated synthesis of copper nanoparticles has been noted to exhibit effective antimicrobial activity against disease-causing pathogenic bacteria (RajeshKumar & Rinitha, 2018). In a study, metal oxide nanomaterials (zinc oxide (ZnO), magnesium oxide (MgO) and ZnO:MgO and ZnO:$Mg(OH)_2$ composites prepared under different synthesis conditions from tropical fruits such as avocado (Persea americana) and papaya (Carica papaya) were investigated antifungal activity was evaluated against *Colletotrichum gloeosporioides* strains. Ultimately, all nanoparticles (NPs) at the tested concentrations significantly inhibited the germination of conidia and caused structural damage in fungal cells (la Rosa-García., 2018). In another study, silver, gold, and silver-gold alloy nanoparticles were biosynthesized using Persea americana fruit peel aqueous extract for their biomedical properties. The biosynthesized nanoparticles showed significant antimicrobial effects against ten clinical bacterial isolates (such as *Proteus vulgaris*, *Listeria monocytogenes, B. Subtilis, E. coli*) tested at different concentrations of 20–80 µg/ml. The synthesis alloy showed consistently higher percent inhibition against E. coli than other synthetic nanoparticles (Adebayo et al., 2019).

4.CONCLUSIONS

Our study confirms previous studies that evaluated the antibacterial effect of avocado. The phytochemical content of avocado fruit is quite high. For this reason, it has been shown in many studies to be potentially beneficial to health. In particular, it has anticancer, antibacterial, cholesterol-lowering and anti-inflammatory effects. The bioactive compounds of avocado have been reported in several studies to be used in the prevention and treatment of oxidative stress and age-related degenerative diseases. For this reason, it is important to evaluate the different parts of the avocado as edible, inedible, ripe or immature. However, in our study, enrichment of avocado with engineered nanoparticles, especially the interaction of the seed part, showed a significantly higher synergistic effect than the pericarp and exocarp, and showed higher antibacterial activity. Thus, in line with the results we obtained, we thought that the already existing antibacterial effect of avocado can be further increased with nanoparticles and used as an antibacterial agent.

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ETHICAL STANDARDS

Conflict of Interest: I declare that I have no conflict of interest with any person or persons.

Ethics committee permission is not required for this study.

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