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Antibacterial and anthelmintic effect of the combination of pomegranate peel and olive leaf extracts

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ABSTRACT

Pomegranate peel and olive leaf are known to have antibacterial activity. These two plants have been studied in various studies. However, there is no study on the synergistic effect of these two plants. In this study, the antibacterial activity of a supplement containing pomegranate peel and olive leaf extracts against *S. mutans* and its antihelmintic activity against *C. elegans* were determined. Results obtained by the disk diffusion method and microdilution test are 12.5 mm and ≥ 1024 µg/mL, respectively. Anthelmintic activity experiments revealed that the lifespan of worms was shortened as a result of the synergistic effect of the extracts. Our results revealed the synergistic effect of these two extracts against the microbes and possible helminths in the oral flora. Increasing antibiotic resistance has led researchers to work on the detection of new plant extracts and substances. It is aimed that this study on plant extracts will help future studies.

Keywords: Pomegranate peel, olive leaves, antibacterial, antihelmintic, *C. elegans*

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INTRODUCTION

Various parts of plants are used to make medicines, cosmetics, and nutraceuticals. The contents of these parts have scientific indications depending on the method of obtaining them[1-3]. The places of plants that are not preferred as nutrients can show special effects and are newly discovered. The peel of pomegranate (*Punica granatum* L.) fruits, which are widely produced by Mediterranean countries including Tunisia, Turkey, Egypt, Spain, Morocco, and Italy, has recently been noticed for its rich and valuable content. Pomegranate peel consists of three parts; exocarp, mesocarp, and pericarp rich in polyphenols such as punicalagin and ellagitannins, gallic acid, and ellagic acid[4]. Punicalin also contains flavone-3-ols, gallotannins, hydroxycinnamic acids, hydroxybenzoic acids, and gallagil esters[5, 6]. It has been observed that these unique ingredients exhibit antioxidant, anti-inflammatory, antiatherogenic, antiangiogenic, antihyperglycemic, and anticarcinogenic effects. They also accelerate wound healing[7, 8]. Antibacterial and antiviral effects of pomegranate peel extracts were also observed[9-12]. It has been determined that pomegranate peel extracts have an antiviral effect against the influenza virus through the inhibition of viral absorption and RNA transcription. Studies show that antiviral activity can also be used against the SARS-CoV-2 virus[13, 14]. Phenolic compounds in the pomegranate peel show activity against Gram negative and Gram positive bacteria. Antimicrobial effects of phenolic compounds were observed to be comparable with those of a chemical antibacterial agent on the tooth[15]. It has been seen that pomegranate peel has important effects in the field of oral and dental health and is a good alternative natural resource to chemical-based applications[16, 17].

Olive leaf is a special medicinal product that is not consumed as a nutrient but contains specific secondary metabolites such as oleuropein and oleacein. Oleuropein, the main component of olive leaf, has anti-inflammatory, antiatherosclerotic, and anti-cancer properties, as well as a strong antioxidant effect with its ability to bind endogenous peptides[18, 19].

Olive leaf extract is a dark brown, bitter-tasting liquid obtained from the leaves of the olive tree (*Olea europaea* L., Oleaceae) native to the Mediterranean region. This leaf extract was found to have antioxidant activity as well as cardioprotective and chemopreventive properties. The main biophenol in the extract is oleuropein, and other biophenols such as verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside are present in lower amounts[20, 21]. The antimicrobial effect of oleuropein has been studied and found to be effective against pathogenic bacteria. It has been found that the antimicrobial activity increases with the phenolic compounds accompanying oleuropein in olive leaf extract[22]. There is

no clear information about the efficacy dose when used in combination with olive leaf extract and pomegranate peel extract. In acute toxicity studies on rats with oleuropein, no deaths or adverse effects were observed despite the administration of a high dose of 1000 mg/kg[23].

Dental caries and dental plaque formation are caused by a mixture of microorganisms and food residues. *Streptococcus mutans* bacteria produce acid in the presence of fermentable carbohydrates such as sucrose and fructose. It especially reproduces on tooth surfaces and damages the hard tooth structure[24, 25]. It has been reported in various studies that *S. mutans*, one of the bacteria that plays a role in the deterioration of dental health, develops resistance to many antibiotics and antimicrobial agents. For these reasons, new drug candidate molecules should be investigated and defined, especially for the treatment of oral infections caused by *S. mutans*[26-28].

Soil-Borne Helminth (STH) infections are caused by intestinal nematodes. Unfortunately, one-fourth of the general population worldwide is infected with STH. These diseases are most common in places like tropical and subtropical regions where fresh water and sanitation are deficient. They cause malnutrition, anemia, retardation of development, and mental problems, especially among school-aged children. In addition to these diseases, oral helminth infestations, including those caused by roundworms, were reported[29]. Resistance to drugs used in the treatment of helminth infections is increasing day by day, as in bacterial infections. Therefore, the discovery of new and safe drugs against pathogenic worms is remarkably important for the pharmaceutical industry. *C. elegans* is a suitable roundworm model organism for preliminary *in vivo* studies due to its many benefits, including its ease of manipulation and cultivation, transparency, short life cycle (2-3 weeks)[30], generation time, tiny size, large hatching size, minimal maintenance costs, cryopreservation, and absence of ethical approval requirements[31]. Moreover, the life cycle of a worm is shortened at 35°C[32]. Due to the brief experimental duration, the thermotolerance assay at 35°C is favored as the initial screening protocol in research on aging and lifespan. Thermotolerance and life span traits in *C. elegans* have been shown to be related[33, 34]. Hence, *C. elegans* is a very useful model organism for discovering new anthelmintic compounds.

In this study, the effectiveness of a commercial dietary supplement, DOLEV Sprey, containing pomegranate peel extract and olive leaf extract, which can be easily used in mouthwash, was investigated against *Streptococcus mutans* and *C. elegans* for determining antibacterial and anthelminthic properties, respectively.

METHODOLOGY

Determination assay of ingredients

Olive Leaf Extract (Tabimer Türkiye) HPLC assay 5,36 % Oleuropein

Pomegranate peel extract (Türkiye)

Determination of Pomegranate Peel Extract

Thermo Orbitrap Q-EXACTIVE (USA)

Mobile Phase A % 1 Formic acid - H_2O

Mobile Phase B % 1 Formic acid – MeOH

Colon: Troyasil C18 HS – 150 x 3 mm 5 µ

Capillary temp. (°C): 320

100 mg/L internal standard solution was added to the extracted sample at a concentration of 3 ppm. The sample was taken through a 0.45 µ filter and taken into a vial. It was analyzed by giving it to the device.

Determination of Olive Leaf Extract

Oleuropein amount were determined in HPLC-PDA/PERKIN ELMER FLEXAR PDA Plus Detector agianst the oleuropein standart of FocusHerb with Purity 80 %. Instrumental analysis conditions were as follows.

Column:	PERKIN ELMER N9303514-ser13120620T COL-AnaIytical C18 Particle size:5µm; Column Length: 250 mm; Inside Diameter : 4,6 mm (5 µm, 250 x 4,6 mm)
Wavelength:	233 nm
Injection:	20 CL
Oven temperature:	2S'C
Time:	30 min
Flow:	1.00mL/min
Mobile phase:	Trifluoroacetic acid: Methanol: Water (1:400:600) Isocritic

Rest of the major ingredients in olive leaf extract were determined as follows.

1 mL of the extract was taken into a 5 mL flask and then a 50% Water - 50% MeOH mixture was added. The mixture was kept in an ultrasonic bath for 15 minutes. It was centrifuged for 5 minutes and the supernatant was taken. The

concentration was adjusted to 3 ppm by adding 100 mg/L of internal standard solution. The sample was taken through a 0.45 μ filter and taken into a vial. Given to device for analysis[35].

Mobile Phase A: %1 Formic Acide – H_2O

Mobil Phase B: %1 Formic Acide- MeOH

Column 3pm Fortis C18 — 150 x 3.0 mm

Commercial Final Formulation

The commercial product formulation contains 20% pomegranate peel extract and 2% olive leaf extract. It also contains xylitol as a stabilizer, benzoic acid and potassium sorbate as a preservative, sucralose as a sweetener, and a nature-identical flavor.

Macroscopic parameters were evaluated on behalf of appearance, final spray volume, pH, and density.

Each puff contains 29.38 mg of pomegranate peel extract and 1.469 mg of olive leaf extract.

Antibacterial activity

Bacterial Strains and Culture Conditions

Streptococcus mutans ATCC 25175 strain was obtained from Ege University, Faculty of Science, Basic and Industrial Microbiology Department. Bacteria were stored at -20 °C in Mueller Hinton Broth (MHB) (Biolife, Italy) supplemented with 20% glycerol.

Then, bacteria were reactivated from stock cultures stored at -20 °C by transferring to Petri dishes containing blood agar and incubating at 37 °C in microaerophilic conditions (95% air and 5% CO_2) for 24-48 hours before assay. The inoculum was prepared as recommended by the Clinical and Laboratory Standards Institute by direct colony suspension method (CLSI, 2012). Colonies of an overnight culture of *S. mutans* were suspended in sterile distilled water and adjusted to 0.5 McFarland standards to reach a final inoculum corresponding to approximately 1 x 10^8 CFU/ml.

Kirby-Bauer Disk Diffusion Test

The antibacterial activities of pomegranate peels and olive leaf extracts were determined by the disc diffusion assay according to the standard method[36, 37]. Briefly, fresh colonies were used to prepare an inoculum at 0.5 McFarland turbidity. The bacterial suspension was streaked using a sterile swab on Mueller

Hinton agar (MHA) plates (Neogen, USA). Paper discs (6 mm diameter) containing pomegranate peels and olive leaf extracts of known concentration (300 µl of 205.6 mg/ml extract) were placed on an MHA plate. Ampicillin discs (10 µg/disc) were used as positive controls and a blank disc (Thermo Fisher Scientific, USA) was used as negative control. Plates were incubated overnight at 37°C, and the antibacterial activity of the spray was expressed by measuring the diameter of the inhibition zone (mm).

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of pomegranate peels and olive leaf extracts was determined against *S. mutans* by using the Clinical Laboratory Standards Institute methods[38, 39]. Briefly, overnight-grown cultures of *S. mutans* were prepared in Brain Heart Infusion Broth (BHIB). The extract was solubilized to 2048 µg/mL in DMSO, and two-fold serial dilutions were prepared in a 96-well microplate (Sarstedt, Germany). Overnight cultures of *S. mutans* strains adjusted to 0.5 McFarland standards ($1x10^8$ CFU/ml) and diluted to 1:10 with BHIB ($1x10^7$ CFU/ml). 5 microliters of the dilution were added to each well to a final density of 5×10^5 CFU/well. A positive control test was performed without an antimicrobial agent and a negative control test was performed without bacteria. The plate was incubated at 37 °C for 24 hours. MIC was defined as the lowest concentration of antimicrobial agent that inhibited the visible growth of the test organism.

Caenorhabditis elegans Survival Assay under Heat Stress

The wild-type strain (N2) *of C. elegans* is provided by the *Caenorhabditis* Genetics Center (CGC), Minnesota. The worms were sustained at 22±2˚C on Nematode Growth Medium following standard procedures[40]. Nematodes were given an OP50-1 *E. coli* strain food source with an optical density (OD) of 0.5. Extracts were added to the L broth containing *E. coli* for the experimental groups. Control-1 contains the same amount of solvent as in the experiment groups, and Control-2 comprises only L broth cultured with *E. coli*. Three plates were prepared for each individual condition (Table 1).

Table 1. The experiment groups information

Groups	Content	Amount	Number of worms
Group A	Pomegranate Peel Extract, Olive Leaf Extract	20 uL in 980 uL E.coli	446
Group B	Pomegranate Peel Extract	20 uL in 980 uL E.coli	555
Group C	Glycerol (Solvent)	20 uL in 980 uL E.coli	462
Group D	-	1000 uL E.coli	320

The tests were conducted on populations of healthy, age-matched, and uncontaminated worms. At the end of the L4 larval stage, exposures were initiated. Prepared petri dishes containing the animals were exposed to room temperature for 24 hours. Subsequently, they were transferred to a pre-heated incubator at 35˚C, and monitored by taking images with a high-resolution scanner (Epson Perfection, V800 Photo) once every 20 minutes till all the worms died. Worms that remained stationary during two consecutive scans were considered to be dead. Utilizing the online application OASIS, survival analysis was performed[35].

RESULTS and DISCUSSION

Determination of Pomegranate Peel Extract

The four highest compounds determined as a result of the analysis are fumaric acid, gallic acid, ellagic acid, and ascorbic acid, respectively (375.02, 109.48, 21.70 and 19.35 mg/L). Uncertainty values are given with the results within the 95% confidence interval.

Determination of Olive Leaf Extract

The major component in olive leaf extract was oleuropein, and its amount was determined as 5,36 % ± 0,09.

Except for oleuropein, the three main compounds were determined as fumaric acid, hederagenin, and caffeic acid, respectively (140.72- 53.22- 21.59 mg/L).

Formulation

Macroscopic evaluation of the final product

Appearance:	Dark Red-brown liquid
Volume:	30 mL
pH (25 °C):	4,15
Density:	1,14 g/cm^3

C. elegans Assay

The combination of the extracts of pomegranate peel and olive leaf decreased nematode lifespan (Figure 1) at 35˚C. There were 320 living worms in Group D, the control group, that were not exposed to any substances. At the ninth scan (after 180 minutes) and under the parameters described, all the worms were dead. In Group C, there were 462 animals treated with extract solvent (glycerol) alone, and all warms were counted dead on the tenth scan (after 200

minutes). In Group B, with 555 live nematodes treated with only pomegranate peel extract, all worms lost their viability on the seventh scan, 140 minutes after treatment. Worms survived until the seventh scan, 140 minutes, in Group A, which contained 446 live worms treated with pomegranate peel and olive leaf extract. It was found that the death rate was higher when both extracts were used together than when only one extract was used.

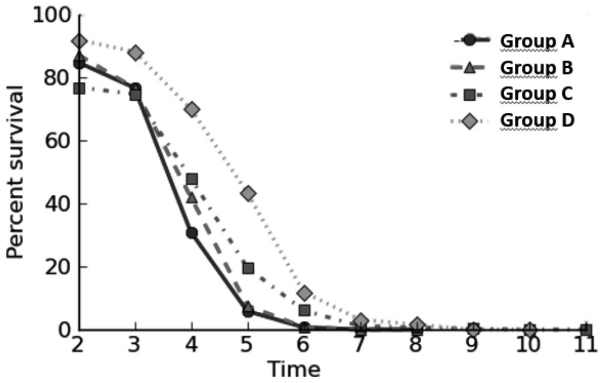

Figure 1. % Survival/Time Curve of experiment groups (1 time interval corresponds to 20 minutes.)

The online application for survival analysis (OASIS) was used to analyze the data[41]. Lifespan data are shown in Table 2. The mean, standard error of the mean, and p-values were determined using the log-rank (Mantel–Cox) method.

Table 2. Restricted mean and % mortality of experiment groups.

Groups	Restricted mean			Age in minutes at % mortality		
	Time intervals	Std. error	95% C.I	50%	75%	90%
Group A	3.99	0.05	3.89 ~ 4.09	100	120	140
Group B	4.14	0.05	4.05 ~ 4.23	100	120	140
Group C	4.27	0.07	4.13 ~ 4.41	80	100	120
Group D	5.10	0.08	4.94 ~ 5.26	100	120	140

The restricted mean is a clinically meaningful representation of average survival or life expectancy over a specific time span beginning at time zero[42]survival analysis has been increasingly used to evaluate prognostic outcomes [1]. Researchers may be familiar with the use of Cox proportional hazards (PH. When comparing the restricted mean values of the groups, it was discovered that

they corresponded with distinct time intervals. The compound with the longest expected lifespan belongs to Group D, which means that all of the formula's components, including the solvent, reduced the lifespan (Table 1). In contrast to the findings reported in the scientific literature[43, 44]. It has been discovered that extracts at these concentrations reduce the lifespan of the worms in our experimental conditions. The fact that the shortest lifespan was observed in the experimental group that received the extracts in combination shows that these extracts may act via distinct mechanisms. In terms of preclinical in vivo toxicity and anthelmintic activity, the findings of this study can help guide future studies and provide new approaches in our fight against oral helminth infestations, especially the ones caused by roundworms that effect people in endemic regions[29].

According to the p-values derived by the Log-Rank test, there was no significant difference between the experimental groups that received the extract combination and those that received simply the pomegranate peel extract. There was a statistically significant difference between all other groups.

Antibacterial Activity

In this study, antibacterial activity of pomegranate peel and olive leaf extract against *S. mutans* was investigated by disc diffusion and MIC tests. The inhibition zone obtained by the disk diffusion method is 12.5 mm (Figure 2). After confirmation of the antimicrobial activity, the minimum inhibition concentration was determined to be ≥ 1024 μg/mL by microdilution test (Figure 3).

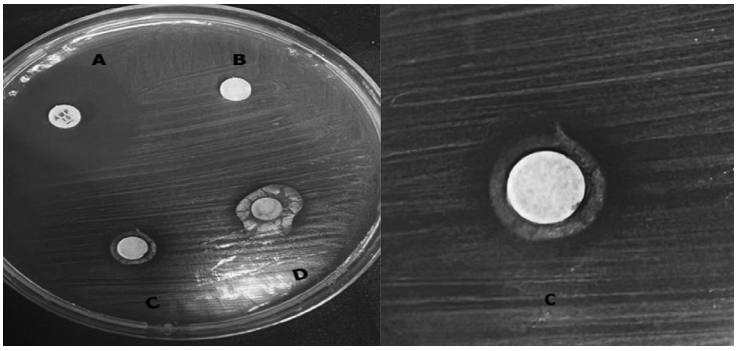

Figure 2. Antimicrobial activity of pomegranate peel and olive leaf extract against *S. mutans* estimated by the disc diffusion method. A=Positive control, B= Negative control, C= 300 ml of spray, D= 600 ml of spray

In another scientific study, the antimicrobial effect of 8 mg/ml and 12 mg/ml concentration of pomegranate peel extract against *S. mutans* was investigated and showed inhibition zone as 9.5 mm[45]. The antibacterial activity of polyphenolic extracts prepared from acacia honey, myrtle leaf and pomegranate peel against cariogenic bacteria in terms of single and synergistic effect was eveluated[46]. They reported that the pomegranate peel extract created an inhibition zone of 16.2 and 11.2 mm (2 mg/disc and 1 mg/disc, respectively) against *S. mutans* and the MIC value was 10 μg/μl. The antimicrobial activity of pomegranate peel against *S. mutans* was evaluated and determined the inhibition zone as 19.75 mm[47]. Pomegranate peel showed higher antimicrobial activity than flower, leaf and stem extracts. In line with these study, we observed remarkably meaningful antimicrobial activity against *S. mutans*.

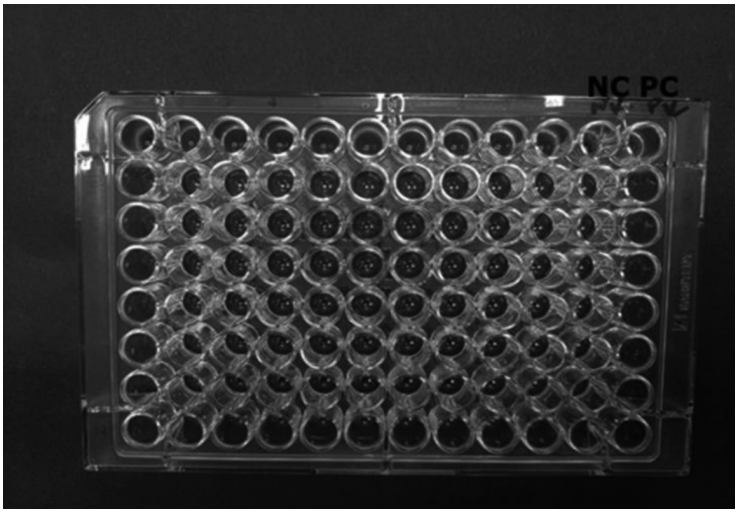

Figure 3. Minimum inhibitory concentrations (MIC) of pomegranate peel and olive leaf extract on S. mutans microbial growth. NC= Negative control, PC=Positive control.

The MICs of olive leaf extracts on *S. mutans* isolates was ranged between 55.80 to 106.8 mg/ml. In addition, the inhibition zone of *S. mutans* (30.3; 27.3; 30.3; 29.5 and 34.3) significantly increased when combined to silver nanoparticles (1:1). In this study, the minimum inhibition concentration (MIC) was determined to be ≥ 1024 μg/mL by microdilution test.

Oral infections have been known to be counterproductive to overall health for over 3000 years. Recent research has added to our understanding of the pathogenic mechanisms linking oral infections to mortality and morbidity. Poor oral health appears to be associated with all forms of mortality, especially among the elderly[48]. This study examines an oral spray containing two distinct plant

extracts; *Punica granatum* and *Olea europaea*. According to studies, pomegranate extract is efficient against pathogenic oral bacteria, gingivitis, plaque, and periodontal disease[49, 50]. The pomegranate peel is the most abundant part of the pomegranate fruit in terms of bioactive compounds[51]. Olive leaf containing phenolic compounds such as oleuropein and hydroxytyrosol has antimicrobial activity[52, 53]. However, there are few studies on the antimicrobial effect of olive leaf on *S. mutans* evaluated the antibacterial effects of olive leaf (aqueous extracts) on *S. mutans* isolates[54, 55]. As the second component of this study, anthelmintic activity is investigated with the help of the model organism, *C. elegans*. It is cost-effective, simple to maintain, and readily available, and has been utilized for research in diverse fields of medicine and biology. Given that it is a nematode, it is not surprising that it is used to find new anthelmintics[56, 57]. The combination of olive leaf and pomegranate peel was tested for the first time in this study and demonstrated significant antibacterial and antihelmintic activity against *S. mutans* and *C. elegans,* respectively.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

AUTHOR CONTRIBUTIONS

Levent Alparslan: Formulation design, supplying of ingredients, evaluation of determination assays, revising manuscript. Betul Giray: Antibacterial activity, revising the manuscript. Meltem Gulec: Anthelmintic activity, revising the manuscript. Nil Kaya: Antibacterial activity, revising the manuscript. Dogan Uvey: Supplying of extracts, revising the manuscript. Abdullah Olgun: Evaluated the biological assay. All authors read and approved the final manuscript.

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