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Investigation of the Protective Effects of Graviola (*Annona muricata*), Moringa (*Moringa oleifera* Lam.) and *Lactobacillus gasseri* in a *Pseudomonas aeruginosa* Fibroblast Wound Model

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Abstract

Objective: This study aimed to investigate the antimicrobial, anti-inflammatory, antioxidant, and anti-apoptotic effects of Graviola (*Annona muricata*), *Moringa oleifera*, and *Lactobacillus gasseri*, alone and in combination, against *Pseudomonas aeruginosa* in vitro wound infection model.

Methods: Antibacterial activity tests against *Pseudomonas aeruginosa* was determined using the Kirby-Bauer disk diffusion method, MIC/MBC, and FIC methods. Biofilm inhibition was measured by crystal violet staining. A scratch wound model using fibroblast cell line was infected with Pseudomonas aeruginosa. Following treatment with the extracts, cell viability, oxidative stress, cytokine levels (IL-1beta, IL-10 via ELISA), and apoptotic gene expression (BAX, BCL-2 via qPCR) were evaluated.

Results: The triple combination (Graviola + Moringa + *Lactobacillus gasseri*) showed the strongest antibacterial activity (23 mm zone; FIC = 0.25) and notable antibiofilm effects. In infected fibroblasts, cell viability increased to with combination treatment, approaching control levels. TOS levels decreased significantly while TAC increased, indicating reduced oxidative stress. IL-1beta levels were significantly lowered, and IL-10 levels were restored. Additionally, BAX gene expression decreased by 47%, and BCL-2 increased by 50% in the triple treatment group, suggesting protection against apoptosis.

Conclusion: The combined application of Graviola, *Moringa oleifera*, and *Lactobacillus gasseri* exerts synergistic antimicrobial, anti-inflammatory, antioxidant, and anti-apoptotic effects against *Pseudomonas aeruginosa* induced wound infection. These results will shed light on the search for alternative natural therapeutic strategies, particularly in the treatment of chronic wounds associated with antibiotic-resistant pathogens.

Keywords: Antibacterial effect, Apoptosis, Biofilm inhibition, Oxidative stress, Wound model

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Graviola (*Annona muricata*), Moringa (*Moringa oleifera* Lam.) ve *Lactobacillus gasseri*'nin *Pseudomonas aeruginosa* Fibroblast Yara Modelinde Koruyucu Etkilerinin İncelenmesi

Öz

Amaç: Bu çalışmada, Graviola (*Annona muricata*), *Moringa oleifera* ve *Lactobacillus gasseri*'nin tekli ve kombinasyon halinde, *Pseudomonas aeruginosa*'ya karşı antimikrobiyal, antiinflamatuar, antioksidan ve antiapoptotik etkileri in vitro yara enfeksiyon modelinde araştırılmıştır.

Yöntemler: *Pseudomonas aeruginosa*'ya karşı antibakteriyel aktivite testleri; Kirby-Bauer disk difüzyon yöntemi, MIC/MBC ve FIC yöntemleri kullanılarak belirlenmiştir. Biyofilm inhibisyonu kristal violet boyama yöntemiyle değerlendirilmiştir. Fibroblast hücre hattı kullanılarak oluşturulan çizik yara modeli *Pseudomonas aeruginosa* ile enfekte edilmiştir. Tedavi sonrası hücre canlılığı, oksidatif stres, sitokin düzeyleri (IL-1beta, IL-10, ELISA ile) ve apoptotik gen ekspresyonları (BAX, BCL-2, qPCR ile) analiz edilmiştir.

Sonuçlar: Graviola + Moringa + *Lactobacillus gasseri* üçlü kombinasyonu en güçlü antibakteriyel aktiviteyi (23 mm zon; FIC = 0.25) ve anlamlı biyofilm inhibisyonunu göstermiştir. Enfekte fibroblastlarda, kombinasyon tedavisi ile hücre canlılığı artmış ve kontrol düzeylerine yaklaşmıştır. TOS seviyeleri anlamlı şekilde düşerken, TAC artmıştır; bu durum azalan oksidatif stresi göstermektedir. IL-1beta düzeyleri önemli ölçüde azalmış, IL-10 seviyeleri ise normale dönmüştür. Ayrıca, BAX gen ekspresyonu %47 azalmış, BCL-2 ise %50 artmıştır; bu durum apoptozisin önlendiğini göstermektedir.

Tartışma: Graviola, *Moringa oleifera* ve *Lactobacillus gasseri*'nin kombinasyonu, *Pseudomonas aeruginosa* indüklenmiş yara enfeksiyonuna karşı sinerjik antimikrobiyal, antiinflamatuar, antioksidan ve antiapoptotik etkilere sahiptir. Bu bulgular, özellikle antibiyotik dirençli patojenlerle ilişkili kronik yaraların tedavisinde alternatif doğal bir terapötik strateji arayışına ışık tutacaktır.

Anahtar kelimeler: Antibakteriyel etki, Apoptozis, Biyofilm inhibisyonu, Oksidatif stres, Yara Modeli.

INTRODUCTION

Pseudomonas aeruginosa is a common gram-negative bacterium that often colonizes in chronic wounds and can disrupt wound healing[1,2]. It is often an opportunistic pathogen that infects individuals with weakened immune systems and people with underlying medical disorders. Diabetes, cystic fibrosis, advanced HIV infection, organ transplant recipients using drugs that suppress the immune system develop chronic wounds, and as a result, Pseudomonas aeruginosa colonizes[3]. *Pseudomonas aeruginosa* can produce various virulence factors and surface proteins that adversely affect wound healing. it's usually resistant to antibiotics, which makes it more difficult to treat[4]. *Pseudomonas aeruginosa* is one of the most commonly isolated bacteria from chronic wounds along with Staphylococcus aureus[3]. Its prevalence varies depending on the geographical region; higher rates have been in South America, Asia and Africa compared to North America and Europe. Larger wound size, longer wound duration, previous amputations, and the use of active wound bandages are associated with greater detection of *Pseudomonas aeruginosa*[1]. The treatment approach used during the progression to a chronic stage is very important. Antibiotic resistance and antibiotic film activity of *Pseudomonas aeruginosa* have led to the search for alternative therapies. Graviola (*Annona muricata*) has been studied for potential wound healing properties using leaves and extracts. Graviola leaves contain compounds that increase antioxidant activity in wound tissues[5]. Studies have shown that topical application of ethyl acetate extract from Graviola leaves increases the activity of essential antioxidants such as catalase and superoxide dismutase, which help reduce oxidative stress in wound healing environments. The extract also supports the upward regulation of the heat shock protein 70 (Hsp70), which plays a critical role in cellular protection and reduction of inflammation[6,7]. This suggests that Graviola may help regulate inflammatory responses during the healing. Macroscopic and microscope analyses of

wounds treated with Graviola extracts show advanced healing characterized by improved tissue regeneration and reduced scar footprint formation. Histological evaluations show better organization of collagen fibers and reduced inflammatory cell infiltration compared to control groups[6]. *Moringa oleifera* is a plant known for its numerous health benefits, including its potential to support wound healing[8]. Studies have shown that various parts of the Moringa plant, especially its leaves, contain bioactive compounds that contribute to improving the wound healing. Moringa leaves exhibit important anti-inflammatory effects, which can help reduce swelling and pain associated with wounds. This feature is very important in the inflammatory phase of wound healing[9]. Moringa is rich in antioxidants, which play a vital role in neutralizing free radicals and reducing oxidative stress in wound tissues. This can facilitate better results of healing by protecting cells from damage during the healing. Studies have shown that Moringa extracts can support the proliferation and migration of fibroblasts, which are necessary for wound closure and tissue regeneration. Specifically, the ethyl acetate fraction of Moringa leaves has increased the viability, migration capacity, and wound healing rates of normal human skin fibroblasts[10]. Moringa contains a variety of phytochemicals, including flavonoids, saponins, and tannins, which contribute to its antimicrobial and wound healing properties. These compounds can help prevent infections and support the healing by promoting tissue repair[11]. A probiotic bacterium, *Lactobacillus gasseri*, has shown promising potential in supporting wound healing and preventing infections, especially in chronic and hard-to-treat wounds. *Lactobacillus gasseri* suppresses the production of pro-inflammatory cytokines such as TNF-alfa and IL-6 in a dose-dependent manner. This anti-inflammatory effect is not specific to *Helicobacter pylori* infections and can also reduce inflammation in host cells induced by lipopolysaccharides (LPS) or lipoteichoic acid (LTA)[12]. *Lactobacillus gasseri* supernatant (LgCS) can inhibit the growth of *Pseudomonas aeruginosa*, prevent the development of biofilm, and partially eliminate advanced biofilms. The topical application of LgCS reduced mortality and prevented systemic spread (sepsis) in mice models with thermal injury and dorsal extraction infected with *Pseudomonas aeruginosa*. *Lactobacillus gasseri* increased the migration of mesenchymal gingival stem cells (GMSCs) in vitro, the expression of stem cell markers, osteogenic differentiation and proliferation[13]. Local injection of *Lactobacillus gasseri* extract supported wound healing in mouse models, and significant differences were observed compared to control groups. *Lactobacillus gasseri* accelerates wound healing in mice through the PI3K/AKT/beta-catenin/TGF-beta1 signal path. The anti-inflammatory and antimicrobial properties of *Lactobacillus gasseri* contribute to its beneficial effects on wound healing by reducing inflammation and preventing infections[14].

Based on this information, we evaluated the single and combined antibacterial effects of Graviola (*Annona muricata*), Moringa (*Moringa oleifera* Lam.) and *Lactobacillus gasseri* against developing *Pseudomonas aeruginosa* in the wound model that we created, cell vitality, cytotoxicity, IL-10, IL1-beta cytokine levels and BAX, BCL-2 gene expression levels.

METHODS

Ethical Statement

Commercially available bacterial strain was used in this study. Therefore, no ethical committee approval was required.

Experimental Design

This experimental design aims to evaluate the potential healing effects of a combination of Graviola extract, *Lactobacillus gasseri*, and

Moringa (*Moringa oleifera* Lam.) in a pathogen-induced wound model. In the first phase, these biological agents were incubated together to obtain fermentation products. Subsequently, this biological combination was applied to infected cell cultures. Wound formation caused by pathogen infection was observed on the cell model created, and the relevant treatment groups were applied to this model. After treatment was applied to the cells, parameters such as mitochondrial ROS production, cell proliferation, and immune response markers were evaluated at the cellular level, using ELISA, other biochemical analyses, and molecular analyses. This design enables the testing of the therapeutic potential of natural and probiotic-based combinations against infection-induced cellular damage under in vitro conditions (Figure 1).

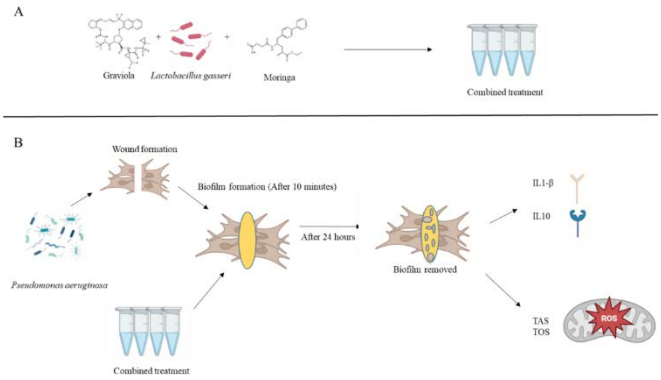

Figure 1. Experimental Design

Isolation of *Pseudomonas aeruginosa* and *Lactobacillus gasseri*

Pseudomonas aeruginosa ATCC 27853 was incubated for 24 hours in a brain-heart infusion broth at 37 °C. It was then planted using the eosin methylene blue and 5% sheep-blood medium planting method. It was again incubated for 24 hours at 37 °C. *Lactobacillus gasseri* Lauer and Kandler ATCC 33323 was purchased American Type Culture Collection. *Lactobacillus gasseri* was cultured in Man, Rogosa, and Sharpe broth (MRSB) at 37 °C under anaerobic conditions for 48-72 hours. The colonies were collected by centrifugation at 5000 x g for 5 minutes. The final amount of *Lactobacillus gasseri* 10^8 cfu/mL was tested for its effectiveness against *Pseudomonas aeruginosa*. It was then stored in tubes containing 50% (v/v) glycerol at -80 °C from the reproducing colonies.

Antimicrobial Susceptibility Testing of *Pseudomonas aeruginosa*

Antimicrobial susceptibility testing was performed using the disk diffusion technique. Briefly, 20 µL of 0.5 McFarland (10^8 CFU/mL) Lactobacillus gasseri bacterial strains were impregnated onto prepared 6 mm sterile disks. Graviola (*Annona muricata*), Moringa (*Moringa oleifera* Lam.), and prepared combinations of Graviola (Annona muricata) + Moringa (*Moringa oleifera* Lam.), Graviola (*Annona muricata*) + *Lactobacillus gasseri*, Moringa (*Moringa oleifera* Lam.) + Lactobacillus gasseri, and Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.) + *Lactobacillus gasseri*. Inhibition zone diameters were then evaluated. The test was incubated on Muller Hinton agar medium (MH, Oxoid) for 24 hours at 37 ° C. The analysis performed was performed using the current interpretation criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI)[15].

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Fractional Inhibitory Concentration (FIC)

Minimum Inhibitory Concentration was determined using the broth microdilution test method using Mueller Hinton Broth (MHB) (Millipore Sigma) medium. The *Lactobacillus gasseri* bacterial strain used in the study was prepared as 0.5 McFarland (10^8 CFU/mL) at a dilution ratio of 1000-1.95 µg/mL. Briefly, 100 µL of MHB medium and 50 µL of agents, including another Lactobacillus gasseri

bacterial strain at concentrations ranging from 1000-1.95 µg/mL, were added to the wells. *Pseudomonas aeruginosa* was also seeded at 0.5 McFarland turbidity scale (10^8 CFU/mL) at 50 µL per well and incubated at 37°C for 24 hours. After incubation, wells where bacterial growth was inhibited were identified. Wells with a higher concentration than those where bacterial growth was inhibited were inoculated onto 5% blood agar. Minimum bactericidal concentrations were determined in media where colonies were found to grow up to 5. Additionally, the functional inhibitory concentrations of agents at concentrations of 1000–1.95 µg/mL and their synergistic, additive, and antagonistic activities in the wells were also evaluated[15].

Determination of Antibiofilm Activity

After determining the MIC, 0.1% crystal violet was added to the wells and incubated for 15 minutes at room temperature. The samples were then stained and washed with PBS. After adding 33% (vol/vol) glacial acetic acid to the wells, spectrophotometric measurements were taken at 595 nm using a microplate reader (Sunrise™, TECAN, Switzerland). Antibiofilm activity was determined using the formula [(OD (control) - OD (test) / OD (control)) x 100] to evaluate the measurements obtained[15].

Cell Culture

Fibroblast Cell Culture

For our study, fibroblasts (PCS-201-010™) have been obtained from ATCC, United States. Cells were stored in liquid nitrogen and the cell suspension was centrifuged for 5 min. Cells were resuspended with fresh medium, 10% Fetal bovine serum (FBS, Sigma Aldrich, St. Louis, MO, USA), and 1% antibiotics (penicillin, amphotericin B, and streptomycin, Sigma Aldrich, St. Louis, MO, USA). Trypsin (Sigma Aldrich, St. Louis, MO, USA) treatment and another centrifugation were performed on cells that were 80% confluent. Cells were seeded in 96-well plates (Corning, USA) and stored in an incubator (5% CO_2; 37 °C).

Infection Wound Model

A 24-well plate was used to cultivate the wound-tested fibroblast cell line until it reached 100% confluence. Following the fifth day, a sterile plastic pipette tip (yellow tip, 100 µl) was used for scraping each well. Phosphate buffered saline (PBS, Sigma Aldrich, St. Louis, MO, USA) was used to aspirate cell material. To create an infection model, cells were infected with *Pseudomonas aeruginosa* at a concentration of 10^8 CFU/mL. To eradicate the biofilm, the cells received treatment with Graviola, Moringa, and *Lactobacillus gasseri*. Using an inverted microscope (Leica Microsystems, Wetzlar, Germany). Every experiment was carried out in triplicate[15].

3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) Assay

10 µL of MTT solution (Sigma Aldrich, St. Louis, MO, USA) should be added to each well plate, after 48-hour treatment. Following four hours of incubation in a CO_2 incubator, 100 µL of DMSO solution was applied to each well of the plate to dissolve the Formazan crystals.

The Spectrophotometer reader (Thermo Fisher, Porto Salvo, Portugal) was used to measure the density at 570 nm[15,16].

Total Oxidant Status (TOS) & Total Antioxidant Capacity (TAC)

Spectrophotometry was used for assessing TOS and TAC. The commercial kit's instructions (Rel Assay Diagnostics, Gaziantep, Turkey) indicate that the color density directly correlates with the quantity of oxidants and antioxidants in the sample15.

IL-1 beta and IL-10 ELISA

We used the ELISA detection kit (Elabscience, USA) to investigate IL-1 beta and IL-10. The principle of this procedure was that the ELISA

interaction with dinitrobenzoic acid would form a yellow complex. The data were read at a wavelength of 450 nm, and the experiment was carried out following the kit protocol[17].

Quantitative PCR (qPCR)

The RNA of cells was isolated using the High Pure RNA Isolation kit (Roche Diagnostics, Sweden). RNA concentration was measured to equalize all RNA amounts with NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Inc). The Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics) was used to perform RNA samples for cDNA in accordance with the prescribed protocol.

qPCR was performed using primers constructed for BAX, BCL-2, and β-actin. Briefly, 1 µl Primer Probe mix, 4 µl of cDNA, 10 µl of Fast Start Essential DNA Probes Master mix (Roche Diagnostics), and 5 µl distilled water were added to each tube to be used (final volume is 20 µl). The PCR cycling conditions were as follows: 5 min at 95 °C, 10 sec at 95 °C, and 30 sec at 60 °C, covering a period of 45 cycles. The results were calculated by calculating the 2−ΔΔCq values. The primers (Table 1) used were as follows:

Table I: Primers

β-actin	Forward	5′-ATGGATGACGATATCGCTGCG-3′
	Reverse	5′-CTAGAAGCACTTGCGGTGCA-3′
BAX	Forward	5′-ATGGCAACTGTTCCTGAACT-3′
	Reverse	5′-TTAGGAAGACACAGATTCCAT-3′
BCL-2	Forward	5′-ATGCCTGGCTCAGCACTGC-3′
	Reverse	5′-TTAGCTTTTCATTTTGATCATCA-3′

Statistical Analysis

Results were calculated as mean ± standard error. Statistical comparisons between groups were performed using Tukey HSD test with one-way ANOVA. All calculations for statistical analysis were performed using SPSS 20 software (IMP Corp.). $p<0.05$ value was considered statistically significant in all tests.

RESULTS

Kirby Bauer Disc Diffusion Results

The antibacterial activity of Graviola (*Annona muricata*), Moringa (*Moringa oleifera* Lam.) and *Lactobacillus gasseri* against *Pseudomonas aeruginosa* is shown in Table 2. The inhibitory effect of Graviola (*Annona muricata*) on *Pseudomonas aeruginosa* is 14 mm. This indicates that this plant has a certain antimicrobial activity. The inhibitory effect of Moringa (*Moringa oleifera* Lam.) is 19 mm higher than that of Graviola. *Lactobacillus gasseri* shows the lowest effectiveness with a 12 mm inhibition range. The inhibitory effect of Graviola + Moringa is 21 mm. The combined use of two plants yields a higher result than the activity that each produces alone. Graviola + *Lactobacillus gasseri* shows an inhibition area of 14 mm. Moringa + *Lactobacillus gasseri* offers an inhibition range of 18 mm. The effect of Moringa also makes a significant contribution here. Graviola + Moringa + *Lactobacillus gasseri* shows 23 mm with the highest inhibition region. This suggests that when the three components are used together, they increase their effect on *Pseudomonas aeruginosa*.

Table II: Antimicrobial Susceptibility Testing of *Pseudomonas aeruginosa* Results

Strain	Inhibition Zones (mm)						
	Graviola (*Annona muricata*)	**Moringa (*Moringa oleifera* Lam.)**	***Lactobacillus gasseri***	**Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.)**	**Graviola (*Annona muricata*) + *Lactobacillus gasseri***	**Moringa (*Moringa oleifera* Lam.) + *Lactobacillus gasseri***	**Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.) + *Lactobacillus gasseri***
Pseudomonas aeruginosa	14	19	12	21	14	18	23

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Fractional Inhibitory Concentration (FIC) Results

MIC and MBC values of Graviola (*Annona muricata*), Moringa (*Moringa oleifera* Lam.) and *Lactobacillus gasseri* against *Pseudomonas aeruginosa* are given in Table 3. The effect of Graviola on *Pseudomonas aeruginosa* shows that it can inhibit bacterial growth at 500 µg/mL, but can kill bacteria at 1000 µg/mL. Moringa has equal MIC and MBC values (1000 µg/mL), indicating that it requires a high concentration for both inhibition and killing of *Pseudomonas aeruginosa*. *Lactobacillus gasseri* had the lowest MIC and MBC values, indicating that this bacterium has the most effective antimicrobial effect against *Pseudomonas aeruginosa*. The ability to inhibit growth at 125 µg/mL and kill bacteria at 250 µg/mL emphasises the potential benefits of probiotic.

Table III: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Results

Antibacterial Activity						
Strain	MIC (µg/mL)			MBC (µg /mL)		
	Graviola (*Annona muricata*)	Moringa (*Moringa oleifera* Lam.)	*Lactobacillus gasseri*	Graviola (*Annona muricata*)	Moringa (*Moringa oleifera* Lam.)	*Lactobacillus gasseri*
Pseudomonas aeruginosa	500	1000	125	1000	1000	250

Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.), Graviola (*Annona muricata*) + *Lactobacillus gasseri*, Moringa (*Moringa oleifera* Lam.) + *Lactobacillus gasseri* and Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.) + *Lactobacillus gasseri* are shown in Table 4. The combination of Graviola + Moringa FIC Index shows a synergistic effect (0.48). The combination of Graviola + *Lactobacillus gasseri* FIC Index has the highest synergy value (0.30). Moringa + *Lactobacillus gasseri* FIC Index This combination also shows a synergistic effect (0.36). Graviola + Moringa + *Lactobacillus gasseri*, FIC Index 0.25 This triple combination has the lowest FIC value and shows the highest synergistic effect. This reveals that the effect of all components on Pseudomonas aeruginosa is further enhanced when used together.

Table IV: Fractional Inhibitory Concentration (FIC) Results

Antibacterial Activity				
Strain	FIC index			
	Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.)	Graviola (*Annona muricata*) + *Lactobacillus gasseri*	Moringa (*Moringa oleifera* Lam.) + *Lactobacillus gasseri*	Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.) + *Lactobacillus gasseri*
Pseudomonas aeruginosa	(0.48) Synergistic	0.30 (Synergistic)	(0.36) (Synergistic)	(0.25) (Synergistic)

Antibiofilm Activity Results

The antibiofilm activity of the agents against *Pseudomonas aeruginosa* is shown in Table 5. Graviola (*Annona muricata*) showed the highest inhibition of 53% at 1000 µg/mL. The inhibition rate decreases as the concentration decreases, but still shows a significant effect of 42.9% at 500 µg/mL. The highest inhibition of Moringa (*Moringa oleifera* Lam.) is observed at 1000 µg/mL with 48.6%. However, the inhibition rate decreases to 27.4% at 500 µg/mL. *Lactobacillus gasseri* provides effective inhibition at 1000 µg/mL with 51.3%. However, it decreases to

41.9% at 500 µg/mL. Graviola + Moringa gave the highest inhibition of 45.9% at 1000 µg/mL. The combined use of the two herbs shows an effect similar to the effect of Graviola alone. The combination of Graviola + *Lactobacillus gasseri* provides an effective inhibition at 1000 µg/mL with 49.1%. Moringa + *Lactobacillus gasseri* provides inhibition at 1000 µg/mL with 47.8%. The triple combination (Graviola + Moringa + *Lactobacillus gasseri*) showed the lowest inhibition at 1000 µg/mL with 46.7%.

Table V: Percent inhibition (%) of biofilm formation of *Pseudomonas aeruginosa* at different concentrations

Percent inhibition (%)										
Agents	Concentrations (µg/mL)									
	1000	500	250	125	62.5	31.25	15.63	7.81	3.90	1.95
Graviola (*Annona muricata*)	53	42.9	36.2	27	18.3	17.9	10.1	8.4	7.3	5.2
Moringa (*Moringa oleifera* Lam.)	48.6	27.4	25.5	21	19.1	17.6	13.9	7.7	6.1	4.1
Lactobacillus gasseri	51.3	41.9	33.6	19.7	14.7	7.9	6.8	7.1	5	5.6
Graviola (*Annona muricata*)+Moringa (*Moringa oleifera* Lam.)	45.9	43.4	38.4	38	27.1	22.3	13.8	10.9	7.3	4.2
Graviola (*Annona muricata*) + *Lactobacillus gasseri*	49.1	44.4	32.1	27.6	23.1	27.4	12.9	8.8	7.6	3.4
Moringa (*Moringa oleifera* Lam.)+*Lactobacillus gasseri*	47.8	42.6	33.8	29.9	21.4	26.2	18.9	15.4	9.6	4.9
Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.)+*Lactobacillus gasseri*	46.7	34.6	29.9	21.3	15.8	14.3	6.5	4.3	3.2	1.9

Cell Culture Results

MTT Assay

The MTT test was applied to test cell viability. The MTT test results are shown in Figure 2. A hospital infection model was attempted to be created by adding *Pseudomonas aeruginosa* to the wound model. When applying the treatment groups, the possible effects of antibiotics were eliminated by preparing an environment without antibiotics. Cells infected with *Pseudomonas aeruginosa* were compared with the control group. The survival rates of the other groups were also compared with the *Pseudomonas aeruginosa* group. The cell viability of the control group was accepted as 100%. An increase of approximately 65% was observed in cell viability because of the infection model ($p<0.0001$). This viability rate is a clear indicator of bacterial infection. High viability rate was detected due to fibroblast cells and *Pseudomonas aeruginosa* colonies in the medium. Graviola and moringa were given separately and in combination with *Lactobacillus gasseri* at therapeutic concentrations. Graviola and Moringa did not significantly affect cell viability compared to *Pseudomonas aeruginosa* alone. Compared to the control group, the cell viability of the Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.) 62.5 µg/mL + *Lactobacillus gasseri* 15 µg/mL group was determined to be 115%. When the findings were examined, it was seen that the damage caused by infection was eliminated and cell viability was preserved.

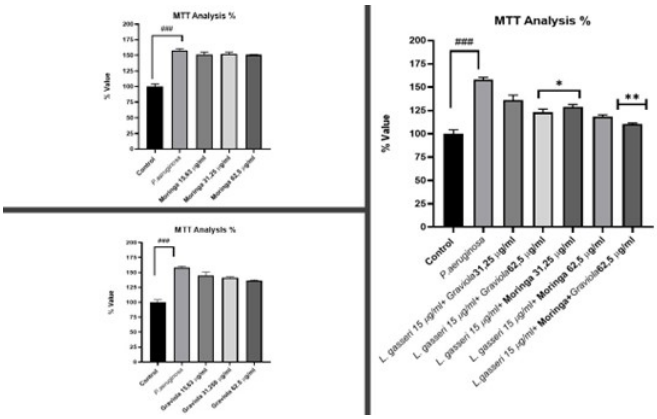

Figure 2. The cell viability was established via the MTT test (n = 12). This study established an infection model of fibroblast cells with *Pseudomonas aeruginosa* by treating them with Graviola. Moringa ve *Lactobacillus gasseri* at various concentrations. The *Pseudomonas aeruginosa* group was compared with the control group. Other groups were compared with *Pseudomonas aeruginosa* and evaluated statistically. The results are

represented by taking the median of three separate investigations (### p <0.0001; * p <0.05; ** p <0.01; *** p <0.001).

Biochemical Analysis Results

To measure the oxidative damage mechanisms of the *Pseudomonas aeruginosa* infected cells, TAC and TOS levels were examined. No oxidative damage was detected in the control group (2 H2O2 equiv/mmol). The TOS levels of *Pseudomonas aeruginosa* infected cells increased approximately 7-fold (p <0.0001) (Figure 3). This was a clear indication that the cells had a high level of oxidative damage. Interestingly, proportional decreases were observed in the treatment groups. Statistically significant changes occurred especially in the combination groups. A significant change occurred in the TOS level in the Graviola + Moringa 62.5 µg/mL+*Lactobacillus gasseri* group. The TOS level, which increased to 13 H2O2 equiv/mmol with *Pseudomonas aeruginosa* infection, approached the control group in this group. TOS level in the Graviola+ Moringa 62.5 µg/mL+ *Lactobacillus gasseri* 15 µg/mL group was determined to 6 H2O2 equiv/mmol (p <0.01).

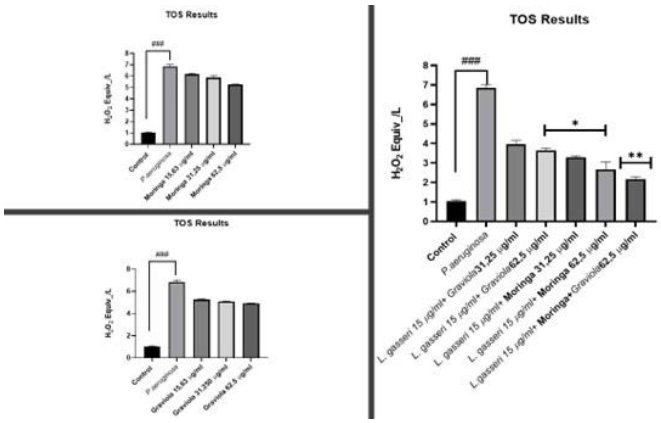

Figure 3. The cell oxidative damage was established via the TOS (n =3). This study established an infection model of fibroblast cells with *Pseudomonas aeruginosa* by treating them with Graviola. Moringa. and *Lactobacillus gasseri* at various concentrations. The *Pseudomonas aeruginosa* group was compared with the control group. Other groups were compared with *Pseudomonas aeruginosa* and evaluated statistically. The results are represented by taking the median of three separate investigations (### p <0.001; * P <0.05; ** p <0.01).

Contrary to TOS measurements, TAC levels were high in the control group, as expected. Oxidative stress levels increased, and antioxidant capacity decreased with *Pseudomonas aeruginosa* infection. TAC levels of infected cells decreased to 2.92 mmol Trolox equiv/L. Antioxidant levels gradually increased in the treatment groups. In the Graviola+ Moringa 62.5 µg/mL + Lactobacillus gasseri 15 µg/mL group, this value reached 8.01 mmol Trolox equiv/L (Figure 4).

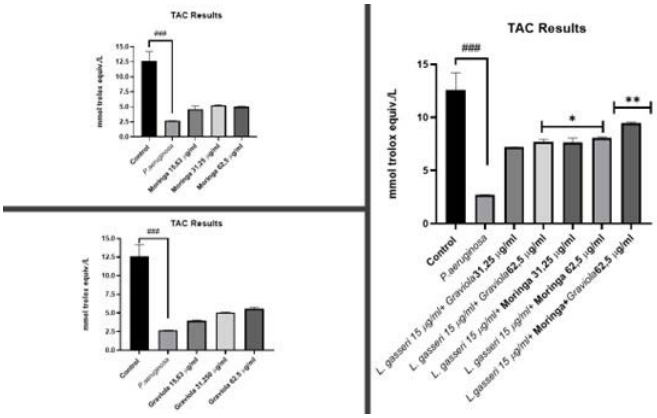

Figure 4. The cell oxidative damage was established via the TAC (n =3). This study established an infection model of fibroblast cells with *Pseudomonas aeruginosa* by treating them with Graviola. Moringa and *Lactobacillus gasseri* at various concentrations. The *Pseudomonas aeruginosa* group was compared with the control group. Other groups were compared with *Pseudomonas aeruginosa* and evaluated statistically. The results are represented by taking the median of three separate investigations (###p <0.001; * p <0.05; ** p <0.01).

IL-1 beta and IL-10 ELISA Results

IL-1beta levels as an inflammation marker and IL-10 levels were determined with ELISA because it has anti-inflammatory properties. When the results were analyzed, IL-1beta levels were calculated as 41.19 pg/mL in the control group and 127.15 pg/mL in the bacterial infection group. Our results show the extent of infection in the diabetic wound model and the resulting increased IL-1beta level. With the applied treatment, the inflammation

disappeared and approached the control group. Among the treatment groups, Graviola (*Annona muricata*) + Moringa (*Moringa oleifera* Lam.) + *Lactobacillus gasseri* approached the control group with 56.5235 pg/mL, and its therapeutic effectiveness came to the fore (Figure 5).

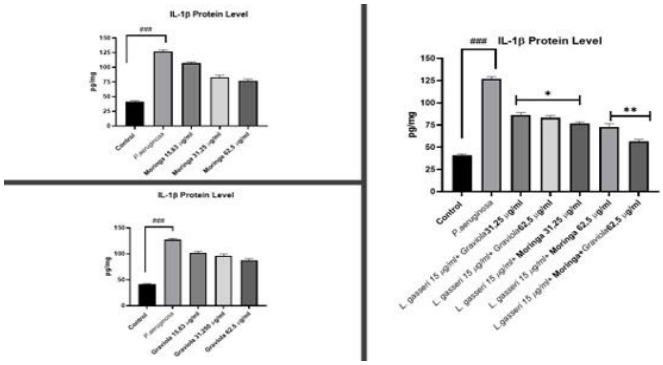

Figure 5. The inflammation damage was established via the IL-1beta (n=3). This study established an infection model of fibroblast cells with *Pseudomonas aeruginosa* by treating them with Graviola. Moringa and *Lactobacillus gasseri* at various concentrations. The *Pseudomonas aeruginosa* group was compared with the control group. Other groups were compared with *Pseudomonas aeruginosa* and evaluated statistically. The results are represented by taking the median of three separate investigations (*p<0.05. **p<0.01. ###p<0.001).

In the control group, the IL-10 level was found to be 158.205 pg/mL. After infection with bacteria, the IL-10 level decreased (59.955 pg/mL). When the infected group and the treatment groups were compared, the Graviola + Moringa + *Lactobacillus gasseri* group, which was close to the control group (122.05 pg/mL), stood out with its therapeutic efficacy (Figure 6).

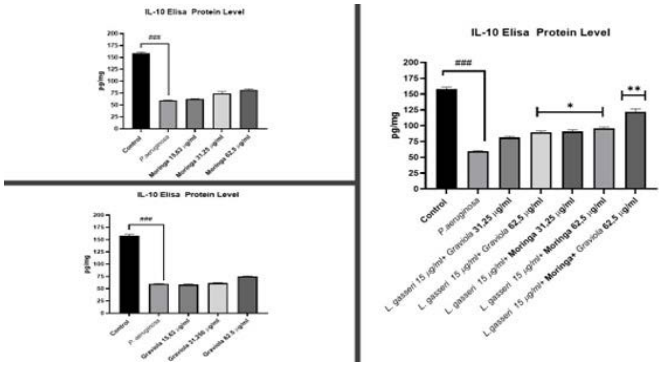

Figure 6. The inflammation damage was established via the IL-10 (n =3). This study established an infection model of fibroblast cells with *Pseudomonas aeruginosa* by treating them with Graviola. Moringa. and *Lactobacillus gasseri* at various concentrations. The *Pseudomonas aeruginosa* group was compared with the control group. Other groups were compared with *Pseudomonas aeruginosa* and evaluated statistically. The results are represented by taking the median of three separate investigations (*p<0.05. **p<0.01. ###p<0.001).

BAX and BCL-2 Results

BAX and BCL-2 genetic analyses are shown in Figure 7 and 8. BAX levels increased by 50% in the *Pseudomonas aeruginosa* group compared to the control group. Moringa and Graviola groups did not show a significant decrease alone. In the Graviola + Moringa 62.5 µg/mL + *Lactobacillus gasseri* 15 µg/mL group, a 47% decrease in BAX levels was observed compared to the *Pseudomonas aeruginosa* group (Figure 7).

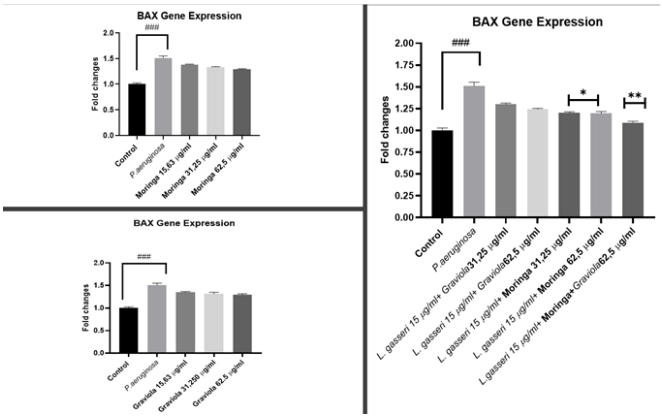

Figure 7. BAX gene levels. an apoptotic marker. *Pseudomonas aeruginosa* group was compared to the control group. The treatment groups were compared to the *Pseudomonas aeruginosa* group (###p<0.001; **p<0.01. *p<0.05).

BCL-2 levels decreased by more than 50% in the *Pseudomonas aeruginosa* group compared to the control group. Moringa and Graviola groups did not show a significant increase alone. In the Graviola + Moringa 62.5 µg/mL + *Lactobacillus gasseri* 15 µg/mL group, an approximately 50% increase in BCL-2 levels was observed compared to the *Pseudomonas aeruginosa* group (Figure 8).

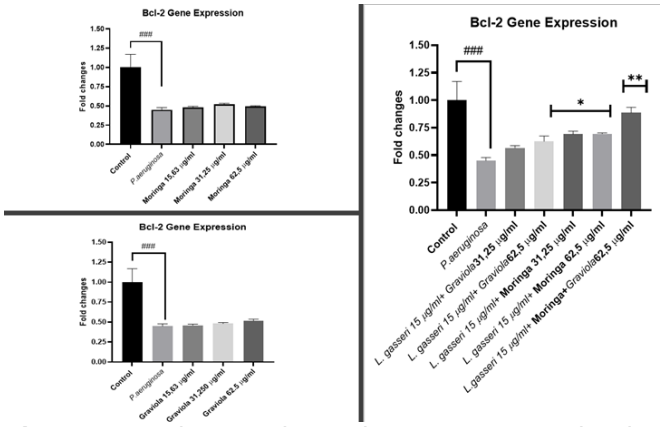

Figure 8. Anti-apoptotic marker BCL-2 gene levels. *Pseudomonas aeruginosa* group was compared to the control group. Treatment groups were compared to the *Pseudomonas aeruginosa* group (###p<0.001; **p<0.01. *p<0.05).

DISCUSSION

Pseudomonas aeruginosa is a bacterium that causes characteristic skin manifestations, such as green nail syndrome, hot tub toe web wounds, and folliculitis[18]. It is known that the extract of *Moringa oleifera* contains some important active phytochemical components such as flavonoids and saponins[19]. Different studies show flavonoids and saponins have antibacterial effects[20]. While saponins in *Moringa oleifera* extract (500 mg) affect the protein permeability of bacteria, flavonoids have been found to inhibit energy metabolism, thus preventing the growth and development of bacteria[21]. In the findings obtained in our study, although the viability rate was shown to be more effective antibacterial at 62.5 µg/mL, it was not significant. The reason for this is that the amount of saponin and flavonoid concentration used was not sufficient. Similar results were obtained from Graviola. Although some of this result is related to the pathogenicity of the bacteria, that it should be tested at a higher rate in our study. *Lactobacillus gasseri* is one of the well-known probiotics. A study about bacteria-bacteria interaction persuades us to evaluate the Lactobacillus effect against *Pseudomonas aeruginosa*. according to the study, Lactobacillus showed beneficial effects against vaginal-origin pathogen bacteria[22].The study demonstrates the same results. *Lactobacillus gasseri* combination of Moringa and Graviola effectively decreased the bacterial population and increased the cell viability ratio consequently. The results support with TOS and TAC results. A decrease in TOS levels shows oxidative stress decreased with phenolic components[23] and also combinations with *Lactobacillus gasseri*[24,25]. TAC results show Graviola and *Lactobacillus gasseri* effectively increase antioxidant capacity but not more Moringa groups. Graviola has strung anti-oxidant activity dose-dependent[26]. Low-dose Graviola combination with *Lactobacillus gasseri* increases the acceptable ratio. IL-1 and IL-10 are cytokines that help us to understand inflammation related to cell oxidative stress. IL-1 is an indicator for inflammation, but IL-10 acts to reduce oxidative stress and anti-inflammatory effect. Jiabing Chen et al (2022) shown IL-10 by regulating mTOR and STAT3 pathways effectively decrease oxidative stress in hepatic cells[27]. The results show a correlation with our study. Especially in moringa high dose combination of probiotics, IL-10 shows a sharp increase. A decrease in oxidative stress inhibits IL-1 expression levels. Ahmed et al. (2010) investigated the relationship between oxidative stress and inflammatory cytokines in nephropathies[28]. They show high oxidative stress stimulates IL-1 and IL-6 secretion. *Pseudomonas aeruginosa* by the induction of oxidative stress and oxidative damage increased expression of IL-1 cytokines. Oxidative stress can promote apoptosis as well. BAX and BCL2 are important mitochondrial markers that can be used for screening cell apoptosis. Stephen J. Wood et al (2015) showed that *Pseudomonas aeruginosa* induces mitochondrial apoptosis (Caspase 3, BAX/BCL-2) in host cells[29]. The results show that BCL-2 expression increased after probiotic and Moringa treatment.

In light of the findings obtained, it is necessary to increase the search for alternative treatments and evaluate the results using more advanced molecular techniques and in vivo studies.

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Author Contributions: Conceptualization, OC, DC, SB, STG and A.T.; methodology, OC, DC,SB, STG and A.T.; formal analysis, OC, DC,SB, STG and A.T.; investigation, OC, DC,SB, STG and A.T.; resources, OC, DC,SB, STG and A.T.; data curation OC, DC,SB, STG and A.T.; writing—original draft preparation, OC, DC,SB, STG and A.T.; writing—review and editing OC, DC,SB, STG and A.T.; visualization OC, DC,SB, STG and A.T.; supervision OC, DC,SB, STG and A.T.; project administration, OC, DC,SB, STG and A.T.

Ethics Committee Approval: Commercially available bacterial strain was used in this study. Therefore, no ethical committee approval was required.

Conflict of Interest: The authors declared no conflicts of interest.

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