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Synergistic *In Vitro* Antimicrobial Activity of Methanolic Extract of Pomegranate Peel and Black Seed

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ABSTRACT

The disc diffusion and agar well diffusion methods were used to investigate the synergistic *in vitro* antibacterial activity of methanolic extracts of pomegranate peel and black seed. Extracts of pomegranate peel and black seed were produced in methanol for this objective and evaluated *in vitro* against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella typhi,* and *Pseudomonas aeruginosa* bacterial strains. The results were then compared to the standard drug gentamycin (50 µg). The synergistic *in vitro* antibacterial activity of pomegranate peel and black seed methanolic extracts revealed varying levels of antimicrobial activity against all test microorganisms.

Keywords: Antimicrobial activity, pomegranate, black seed, synergism, disc diffusion, agar diffusion.

1. INTRODUCTION

The continuing evolution of communicable diseases, as well as pathogenic resistance to existing medications, has accelerated the quest for new novel leads to treat fungal, parasitic, bacterial, and viral infections.[1] Natural plant-derived molecules are still proven to be a vital source of medications for humans, despite previous breakthroughs in drug discovery through molecular modeling, combinatorial, and synthetic chemistry.

Medicinal plants have been utilized to treat diseases in many indigenous systems of medicine and also herbal remedies for generations.[2] Herbal remedies are also made with medicinal herbs since they are regarded to be safer than current allopathic treatments. Combination of herbal cures is one of the tactics used in traditional herbal therapy to overcome these processes in light of new epidemics and the developing resistance in those with current curatives.[3,4]

Herbal treatments are frequently made from a mixture of different plant species. The pharmacological activities of such mixes could be due to a combination of distinct types of chemicals with different mechanisms of action.[5] Synergistic methods of mixing herbal components is responsible for the potential pharmacological effects. Synergism occurs when two or more substances interact in such a way that they mutually increase, magnify, or potentiate each other's effect more than the total of their individual effects.[6]

Plants like the pomegranate (*Punica granatum* L.) were among the earliest to be cultivated by humans. It has been used as an antibacterial agent

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since biblical times. Pomegranate fruit production have been expanding since the beginning of the twenty-first century, owing to an increasing number of scientific research about its health advantages. Many phytochemicals, such as phenolic acids, flavonoids, and tannins, have been found in significant concentrations in the fruits and peel. Due to the presence of hydrolysable tannins, pomegranate by-products, particularly pomegranate peel extract, have recently received increased interest due to scientifically verified medicinal qualities such as antioxidant, antibacterial, antitumor, immunomodulatory, and anti-inflammatory activity. Many investigations on the bactericidal effects of pomegranates on a range of important infectious and drug-resistant strains have been carried out over the years in various parts of the world.[7]

The seeds of black cumin (*Nigella sativa* L.), also known as black seed, have been utilized for therapeutic purposes throughout Asia, the Middle East, and Africa for ages, both as herb and pressed into oil. The constituents of black cumin seed include fixed oil, proteins, alkaloid, saponin, and essential oil, according to extensive research. The active ingredient in black cumin seed is thymoquinone. These seeds are considered as one of the most powerful types of curative medicine in Islam, and they have been included in the Prophet Muhammad's ﷺ medication. Black cumin seeds have been used traditionally in Middle Eastern folk medicine as a treatment for various diseases for more than 2000 years ago. For more than 2000 years, black cumin seeds have been utilized as a therapy for many ailments in Middle Eastern folk medicine. Fungal infections, anemia, intestinal issues, lack of appetite, eye infections, indigestion, rheumatoid arthritis, kidney disease, high blood pressure, innate internal bleeding, loss of consciousness, menorrhagia, breathing problems, pneumonia, migraine, flu, fever, and dermatitis have all been treated with black cumin seeds in traditional Arabic herbal medicine.[8]

Many research work has assessed the antibacterial effects of pomegranate and black seeds against a variety of bacterial and fungal species independently, to our knowledge. Furthermore, no study has documented the synergistic antibacterial impact of pomegranate peel and black cumin seed isolates for antimicrobial activity testing. As a result, the main goal of this work was to explore if a combination of methanolic pomegranate peel and black cumin seed extracts may have a synergistic antimicrobial impact when evaluated *in vitro* for antibacterial potencies.

2. MATERIAL & METHODS

2.1 Drug and Chemicals

Methanol was obtained from Sigma Aldrich, India. Gentamycin obtained from Medplus Health Services Pvt. Ltd. Soxhlet apparatus, digital weighing balance, glassware etc. used were of analytical grade.

2.2 Collection and Extraction of Plant Materials

During the fruit season, fresh pomegranate fruits were harvested from trees in Maharashtra, India. Fruits were harvested by hand, rinsed, and peeled. After a few days of air drying, the peel was crushed. The black seeds were found in a local market Hyderabad, India. In a thimble, 5g of finely ground homogeneous pomegranate peel powder and black cumin seed powder samples were retained in the identical quantities in a permeable pouch produced from cellulose robust filter (paper manufactured physically), and then it was introduced into the Soxhlet chamber. In the bottom flask of Soxhlet, 300 mL methanol was used for extraction. By adding water intake and outflow, the upper half was equipped with a condenser. The methanol solvent was warmed to a comfortable temperature. The hydroalcoholic process was kept going for another 48 hours. residue was collected and then condensed to provide a dry extract for further biological activity investigation.[9]

2.3 Test Microorganisms for Antibacterial Assay

The Bacteriology Unit of Institute of Medical Research (IMR), India have provided the test

organisms. *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella typhi,* and *Pseudomonas aeruginosa* were the human bacterial pathogens employed in this study. All isolates were sub-cultured onto specific culturing media as soon as they arrived to assure purity and confirm their identity. Mueller Hinton Agar (MHA) (Merck, Germany) was used to maintain and test the strains, which was kept at 4°C. Before being utilized in the antibacterial testing, the test organisms were grown overnight at 37°C.[10]

2.4 Anti-Bacterial Assay

2.4.1 Disc Diffusion Method

The disc diffusion technique was used to investigate the antibacterial activity of the crude methanolic peel extract of pomegranate and black seed, both alone and in combination (Kirby-Bauer method). The test chemicals, namely the methanolic peel extract of pomegranate and black seed, were put onto a 0.5 mm (hi-media) disc and left to dry in the agar disc diffusion technique. As a result, the test chemical was totally saturated on the disc at a concentration of 40 mg/mL. The discs were then put directly on the surface of Muller Hinton agar plates, swabbed with the test organism, and incubated for 24 hours at 37°C. The presence of inhibitory zones was determined using a vernier caliper, documented, and taken as a sign of antibacterial activity.

2.4.2 Agar Well Diffusion Method

Muller-Hinton agar plates were made, and 5 mm wells were cut out and swabbed with various cultures. The cut wells were then filled with 50 liters of both aqueous and solvent extracts of flowers and foliage, and the plates were incubated for 24 hours at 37°C. The presence of inhibitory zones was determined using a vernier caliper, documented, and taken as a sign of antibacterial activity.[11]

2.4.3 Statistical Analysis

The findings were evaluated using the statistical approach of standard error of mean (SEM) deviation method.

3. RESULTS

3.1 Plant Extract Yield

Table 1 shows the ethanobotanical data of the plants used, as well as their extract % yield. Plant extract residues varied from 10.54 to 3.92 g after extracting 50 g of dried plant materials with methanol.

3.2 Disc Diffusion Method

Using disc diffusion and agar well diffusion methods, the antibacterial activity of the methanolic peel extract of pomegranate and black seed against different bacterial strains, including two gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and three gram-negative bacteria (*Escherichia coli, Salmonella typhi,* and *Pseudomonas aeruginosa*) were evaluated individually and in combination.

Table 2 shows the results of disc diffusion testing of antibacterial activity of pomegranate and black seed methanolic peel extracts alone and in combination. The gram-positive bacterial strain *S. aureus* was shown to be the most resistant to pomegranate and black cumin individual extracts, with inhibition zones of 15.6±0.31 and 10.5±0.20, respectively. *P. aeruginosa* was determined to be the gram-negative bacterial strain that was inhibited the most by pomegranate and black cumin extracts, with inhibition zones of 15.4±0.27% and 7.6±0.30%, respectively. The findings of the pomegranate and black cumin combination testing demonstrated that the extract was possibly useful in suppressing microbial growth of pathogenic bacteria, with varying efficacy retarding microbial growth of all pathogenic bacteria tested at 10 mg/ml.

Table 2: The ethnobotanical data of the plant species used, and the percentage of extract yielded

Plant Extract	Family	Common Name	Plant Part Used	Extract Yield (%)
Punica granatum	Lythraceae	Pomegranate	Peel	10.54
Nigella sativa	Ranunculaceae	Black Cumin	Seed	3.92
Punica granatum & *Nigella sativa*			Peels & Seeds	7.13

Table 2: Antimicrobial activity of methanolic peel extract of pomegranate and black seed (10 mg/ml) by disc diffusion method

Plant Extract	Inhibition Zone (mm)				
	Gram +ve Pathogenic Bacteria		Gram -ve Pathogenic Bacteria		
	B. cereus	*S. aureus*	*E. coli*	*S. typhi*	*P. aeruginosa*
Punica granatum	15.2±0.28	15.6±0.31	9.8±0.27	5.3±0.18	15.4±0.27
Nigella sativa	5.2±0.21	10.5±0.20	3.2±0.18	5.2±0.25	7.6±0.30
Punica granatum & *Nigella sativa*	13.2±0.34	14.8±0.37	8.5±0.24	4.1±0.28	14.2±0.21
Gentamycin (5µg)	14.3±0.33	17.2±0.24	15.7±0.19	18.2±0.34	13.2±0.27

Data are means of three replicates (n=3) ± standard error of mean

The greatest inhibition zones were reported against gram-positive *S. aureus* and gram-negative *P. aeruginosa* bacteria strains, with 14.8±0.37 and 14.2±0.21, respectively.

3.3 Agar Well Diffusion Method

Table 3 shows the results of disc diffusion testing of antibacterial activity of pomegranate and black seed methanolic peel extracts alone and in combination. The gram-positive bacterial strain *B. cereus* was shown to be the most resistant to pomegranate and black cumin extracts, with inhibition zones of 20.5±0.12 and 12.2±0.21, respectively. The gram-negative bacterial strain *E. coli* showed the greatest suppression by pomegranate and black cumin individual extracts, with inhibition zones of 12.5±0.14 and 8.6±0.24, respectively. The findings of the pomegranate and black cumin combination investigations demonstrated that the extract was possibly useful in inhibiting microbial growth of pathogenic bacteria, with varying efficacy inhibiting microbial growth of all pathogenic bacteria tested at 10 mg/ml. The greatest inhibition zones were reported against gram-positive *B. cereus* and gram-negative *S. typhi* strains, with 11.2±0.14 and 14.2±0.21 inhibition zones, respectively.

4. DISCUSSION

Plants having preventative and therapeutic properties have been used to help meet health-care demands in recent years. There are three key reasons to be interested in plant extract's ability to treat and heal. Pharmacological investigations have shown that many plants contain antibacterial properties. Furthermore, people are becoming more aware of the negative consequences of overprescribing conventional antibiotics. Finally, antibiotic-resistant bacteria are becoming more common with time.[12]

To address these issues, phytochemicals, such as flavonoids, tannins, terpenoids, steroids, polyphenols, terpenes, and alkaloids, are natural active substances found in plants that are used to cure diseases and as a vitamin and dietary supplement.

Table 3: Antimicrobial activity of methanolic peel extract of pomegranate and black seed (10 mg/ml) by agar well diffusion method

Plant Extract	Inhibition Zone (mm)				
	Gram +ve Pathogenic Bacteria		Gram -ve Pathogenic Bacteria		
	B. cereus	*S. aureus*	*E. coli*	*S. typhi*	*P. aeruginosa*
Punica granatum	20.5±0.12	19.5±0.11	12.5±0.14	8.6±0.17	11.5±0.15
Nigella sativa	12.2±0.21	11.2±0.17	8.6±0.24	6.3±0.17	7.2±0.16
Punica granatum & *Nigella sativa*	16.2±0.21	15.3±0.25	10.7±0.15	11.2±0.14	10.3±0.18
Gentamycin (5µg)	15.7±0.21	19.2±0.19	16.3±0.17	17.64±24	12.8±0.26

Data are means of three replicates (n=3) ± standard error of mean

Polyphenols are the major component of flavonoids, and their presence can boost antibiotic activity against microorganisms. Flavonoids are significant and effective antibacterial chemicals that form complexes with bacterium cell walls, proteins, and extracellular components. Terpenoids are implicated in the disintegration of membranous tissue and the weakening of microorganism cell walls. When saponins interact with bacteria, enzyme proteins leak out of the cell. Pomegranate and black seed are two of these plants that play an essential part in traditional medicine.[13,14]

Pomegranate is known for its antimalarial, antimicrobial, fungicidal, antiangiogenic, and anticancer properties, whereas black seed contains thymoquinone, which is one of the most active constituents responsible for broad spectrum antimicrobial actions against gram-positive and gram-negative bacteria, viruses, parasites, and fungi.[15,16]

Plant chemicals can be extracted using a variety of solvents depending on their polarity. Because it is a polar solvent, ethanol inhibited harmful pathogens well, most likely because it removed antimicrobial chemicals from plant components. Microbial effects can also be noticed utilizing medicinal herbs extracted using hexane and petroleum ether, as per Duraipandiyan et al., 2006. Extracts made using hexane, a nonpolar solvent, did indeed include antibacterial components that may suppress methicillin-resistant *S. aureus* in their investigation. Likewise, when we employed a polar solvent in our investigation, we saw some antimicrobial action, although the inhibition zone was less than when we used a nonpolar solvent.[17]

Different microorganisms were utilized in this investigation to test the *in vitro* antibacterial potencies of methanolic pomegranate peel and black cumin seed extracts for putative synergistic antimicrobial effects. Pomegranate peel and black cumin seed methanolic extracts exhibited good effectiveness against *S. aureus* and *S. typhi*. The fast growth in antibiotic resistance of *S. aureus* and *S. typhi* has caused widespread alarm in the medical community. In hospitals, it has become a major endemic pathogen. The correct identification of *S. aureus* and *S. typhi* isolates is also a serious difficulty for the most clinical microbiology laboratories due to the varied nature of methicillin resistance.[18] *S. aureus* isolates are gram-positive bacteria with two layers of membranes: peptidoglycan-based outer layers and cytoplasmic-based inner layers that are easily pierced. For this resistant form of bacteria, new therapies are required, and active chemicals found in natural goods might be the solution. *S. typhi* isolates are gram-negative bacteria that gain resistance by horizontal gene transfer.[19]

5. CONCLUSION

According to the findings of the current study, a combination of methanolic extract of pomegranate peel and black cumin seeds might suppress the formation of human infections. The findings are intriguing, but further testing is required before they can be used in reality, since the most active extracts may be isolated from therapeutic antimicrobials and submitted to further pharmacological examination.

Conflict of Interest: None

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