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*Original Article*

# Synergistic Effect of Pakistani Honey with Black Seeds against Food Borne Pathogens

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## ABSTRACT

Background: Foodborne infections represent a significant global public health issue. Honey has long been utilized not only as a food but for its medicinal properties in gastrointestinal and topical applications, and as a natural preservative. Similarly, black seeds have been employed in traditional medicine and food preservation due to their therapeutic properties. A synergy-based combination of these herbal extracts may enhance antimicrobial efficacy against foodborne pathogens.

Objective: This study aims to assess the antibacterial effectiveness of various Pakistani honey types (Acacia, Ziziphus, and Sidr) in combination with black seeds against common foodborne pathogens.

Methods: The antibacterial activities of Acacia, Ziziphus, and Sidr honey were evaluated using an agar well diffusion assay. This study also examined the synergistic effects of these honeys combined with black seed extracts. Minimum Inhibitory Concentrations (MICs) were determined through the tube dilution method.

Results: The combination of Ziziphus honey and black seeds demonstrated the most significant antibacterial effect, with inhibition zones measuring 25.0 mm for Escherichia coli, 28.0 mm for Salmonella typhi, and 30.0 mm for Staphylococcus aureus. These results indicate that the synergistic effects of honey and black seed extracts are more potent than when each is used separately. The lowest MIC observed was 3.12 mg/ml against Escherichia coli and Staphylococcus aureus, and 6.25 mg/ml against Salmonella typhi.

Conclusion: The findings affirm the traditional use of various Pakistani honeys combined with black seeds in treating foodborne pathogens, promoting health without adverse effects. This natural combination could be further explored as a potent therapeutic strategy against multidrug-resistant pathogens.

Keywords: Agar well diffusion assay, Antibacterial activity, Black seed extract, Foodborne pathogens, Honey extract, Minimum Inhibitory Concentration (MIC), Synergistic effect.

## INTRODUCTION

Foodborne infections remain a significant global health concern, responsible for numerous illnesses and fatalities annually. These infections stem from a variety of pathogens, and although extensive research has been conducted on their management, the primary treatment remains antibiotic therapy (1). However, the widespread misuse and overuse of antibiotics have precipitated the emergence of multidrug-resistant strains, complicating the treatment landscape for foodborne diseases (2). Pathogenic bacteria such as Escherichia coli, Salmonella spp., and Staphylococcus aureus continue to pose significant health risks worldwide (3, 4). The adverse effects associated with synthetic compounds have underscored the need for alternative natural therapies. These alternatives could either stand alone or be used in combination with other natural substances to combat foodborne diseases (5).

Among various natural substances, honey is celebrated for its health-promoting qualities, including its antibacterial activity against a broad spectrum of microorganisms. Its efficacy is attributed to factors such as hydrogen peroxide, low acidity, high sugar concentration, and bee-derived proteins (6, 7). In Pakistan, diverse types of honey such as Sidr, Acacia, Citric, Tasmanian, and Ziziphus have shown potent antimicrobial potential. Despite this, studies specifically examining the antimicrobial activity of these honeys in combination with black seeds against foodborne bacteria are scant (8). Nigella sativa, commonly known as black seed, has also demonstrated antimicrobial properties, making it a valuable component in traditional medicine. Black seeds are rich in bioactive compounds like thymol, thymoquinone, and thymohydroquinone, which contribute to their antibacterial capabilities (9).

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Recent research has increasingly focused on synergistic herbal formulations that combine multiple extracts to enhance therapeutic efficacy. Such synergism is believed to improve the bioavailability of active constituents, mitigate the toxic effects of synthetic agents, and amplify therapeutic outcomes (10). Notably, several studies have documented the enhanced antibacterial potential when black seeds and honey are used together, suggesting that their combined effect could serve as an effective alternative to reduce the adverse effects associated with conventional antibiotics (11, 12).

The objective of this study is to evaluate the effectiveness of honey and black seeds, both independently and in combination, against common foodborne pathogens. This assessment aims to establish whether the synergistic effects of these natural compounds can provide a viable and potent alternative to traditional antibiotic treatments, potentially offering a reduction in side effects and resistance issues.

## MATERIAL AND METHODS

Samples of various types of Pakistani honey—Acacia, Ziziphus, and Sidr—were sourced from honey banks and directly exported from local farms in Pakistan. These honey samples were stored in a dark environment at room temperature. Black seeds were procured from local vendors in Karachi, Pakistan.

For this study, three different foodborne pathogens (FBP)—Escherichia coli, Staphylococcus aureus, and Salmonella typhi—were utilized. These pathogens were initially obtained from the Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories complex in Karachi. Subsequent cultures of these isolates were maintained and preserved at -20 °C for future experiments.

To prepare the ethanolic extracts of honey, 10 grams of each honey type were dissolved in 100 ml of ethanol (w/v). This solution was vigorously mixed using a vortex mixer and subsequently centrifuged at 4000 rpm for 10 minutes. The supernatant was collected, filtered, and the filtrate was evaporated using a rotary evaporator. The dry residue was then reconstituted with 10 mL of dimethyl sulphoxide (DMSO) and stored at room temperature until needed.

Similarly, the black seeds extracts were prepared by dissolving 10 grams of black seeds in 100 ml of ethanol and incubating the solution in a water bath at 37°C for 5-6 hours. Post-incubation, the solution was filtered using Whatman filter paper. The filtrate was placed in an oven at 45°C overnight to evaporate the excess ethanol, resulting in a powdered form of the extract which was stored in an airtight container at room temperature.

The antibacterial activity of both honey and black seeds extracts was assessed using the agar well diffusion method. A log phase culture of each pathogen ($5 \times 10^8$ cfu/ml) was evenly spread on Muller Hinton agar plates. Wells of 5mm diameter were created using a sterilized borer, and 100 µL of each extract was applied to the respective wells. Dimethyl sulphoxide served as the control. Plates were then incubated at 37°C for 24 hours, and the inhibition zones were measured in millimeters.

The synergistic effect of the combined extracts was also evaluated against the selected foodborne pathogens. Honey and black seeds extracts were mixed in a 1:1 ratio (v/v) and tested using the same agar well diffusion assay. Post-incubation, the zones of inhibition around the wells were measured to assess the combined antibacterial activity.

The minimum inhibitory concentration (MIC) was determined using the broth dilution method. A stock solution was prepared by dissolving equal volumes (5 ml) of each extract in DMSO, followed by two-fold serial dilution in nutrient broth tubes to achieve various concentrations (100, 50, 25, 12.5, 6.25, 3.125, and 1.562 mg/ml). A log phase culture (100 µl) of each pathogen was added to the tubes. The tubes included a positive control (broth with pathogen) and a negative control (broth with extract). After 24 hours of incubation at 37°C, the tubes showing no growth at the lowest concentration were identified as the MIC.

Statistical analysis was performed on all experimental data, which were conducted in triplicates and expressed as mean ± standard deviation. Differences between datasets were analyzed using a two-way analysis of variance (ANOVA), followed by a post hoc Tukey's multiple comparison test. A p-value of less than 0.05 was considered statistically significant.

## RESULTS

In the investigation of antibacterial activities, the agar well diffusion method revealed varying degrees of efficacy among honey extracts. Ethanol extracts of Ziziphus honey demonstrated the highest inhibitory action, particularly against Salmonella typhi, producing a zone of inhibition measuring 19.6 mm. This was attributed to the presence of phenolics and essential oils within the honey. Conversely, Sidr honey exhibited modest inhibitory zones, notably 12.0 mm against Staphylococcus aureus, while Acacia honey showed minimal effectiveness, particularly against Gram-negative bacteria, which might be due to its constituent properties.

Further assessment of antibacterial activity focused on black seed extracts, which were also prepared in dimethyl sulphoxide (DMSO) and evaluated using the same diffusion assay. The results indicated that black seed extracts exhibited inhibitory effects against all tested pathogens, with the most pronounced effect against Escherichia coli, where an 18 mm inhibition zone was observed. In contrast, the least activity was recorded against Staphylococcus aureus with a 12 mm zone.

 

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The study also explored the synergistic effects of combining honey extracts with black seed extracts. When mixed in a 1:1 ratio, these combinations showed enhanced antibacterial properties. Notably, the mixture of Ziziphus honey and black seeds yielded the most substantial inhibitory zone of 30.0 mm against Staphylococcus aureus. Meanwhile, the combination of Sidr honey and black seeds increased the zone of inhibition to 22.33 mm, underscoring the potential of synergistic interactions to boost antibacterial efficacy. However, the combination involving Acacia honey and black seeds was less effective against Salmonella typhi. Overall, the combined extracts displayed significantly greater antibacterial potential compared to the individual honey extracts, indicating a robust synergistic effect.

The Minimum Inhibitory Concentration (MIC) of these extracts was quantified using the broth dilution method to ascertain the lowest effective concentrations against the bacteria. The combination of Ziziphus honey and black seeds demonstrated the lowest MIC value of 3.12 mg/ml against both Escherichia coli and Staphylococcus aureus. For Salmonella typhi, the MIC was slightly higher at 6.25 mg/ml. These findings underscore the potent antimicrobial capabilities of the combined extracts, particularly when leveraging the complementary properties of honey and black seeds.

These results collectively suggest that the natural constituents in honey and black seeds, especially when combined, possess significant antibacterial properties, offering a promising alternative to conventional antibiotics. The exploration of these natural compounds could pave the way for developing novel antibacterial therapies aimed at combating resistant strains of foodborne pathogens.

Table 1: Antibacterial activity of different honey extracts against food borne pathogens.

| Indicator Organisms | Zones of Inhibition (mm) | | |
|---|---|---|---|
| | Sidr Honey Extract | Acacia Honey Extract | Ziziphus Honey Extract |
| Escherichia coli | 15.3 ± 0.2 | 8.3 ± 0.4 | 14.0 ± 0.4 |
| Salmonella typhi | 13.3 ± 0.2 | 8.0 ± 0.4 | 19.6 ± 0.3 |
| Staphylococcus aureus | 12.0 ± 0.2 | 7.0 ± 0.2 | 17.0 ± 0.2 |

DMSO = negative control having no zone of inhibition. The mean ± standard deviation values of antibacterial activity of honey extracts at < 0.05.

Table 2: Antibacterial Activity of Black Seeds against Food Borne Pathogens.

| Indicator Organisms | Zones of Inhibition (mm) |
|---|---|
| Escherichia coli | 18.0 ± 0.3 |
| Salmonella typhi | 15.0 ± 0.2 |
| Staphylococcus aureus | 12.0 ± 0.2 |

DMSO = negative control having no zone of inhibition. The mean ± standard deviation values of antibacterial activity of black seeds extracts at < 0.05.

Table 3: Synergistic Effects of Honey and Black Seeds Extracts against Food Borne Pathogens.

| Indicator Organisms | Zones of Inhibition (mm) | | |
|---|---|---|---|
| | Sidr Honey + Black seed | Acacia Honey + Black seed | Ziziphus Honey + Black seed |
| Escherichia coli | 18.0 ± 0.3 | 20.0 ± 0.4 | 25.3 ± 0.3 |
| Salmonella typhi | 17.0 ± 0.2 | 16.0 ± 0.4 | 28.0 ± 0.2 |
| Staphylococcus aureus | 22.3 ± 0.2 | 17.0 ± 0.2 | 30.0 ± 0.4 |

DMSO = negative control having no zone of inhibition. The mean ± standard deviation values of synergistic effect of honey and black seeds extracts at < 0.05.

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Table 4: Minimum Inhibitory Concentration of the synergistic effect of Honey and Black Seeds Extracts

| Indicator Organisms | Minimum Inhibitory Concentration (mg/ml) | | |
|---|---|---|---|
| | Sidr Honey + Blackseeds | Acacia + Blackseeds | Ziziphus + Blackseeds |
| Escherichia coli | 6.25 ± 0.02 | 6.25 ± 0.02 | 3.12 ± 0.04 |
| Salmonella typhi | 12.5 ± 0.25 | 6.25 ± 0.02 | 6.25 ± 0.02 |
| Staphylococcus aureus | 12.5 ± 0.25 | 12.5 ± 0.25 | 3.12 ± 0.04 |

## DISCUSSION

Honey has long been recognized for its antibacterial properties, effectively inhibiting the growth of microbes, a capability documented through centuries of traditional use. The rising incidence of foodborne diseases alongside the prevalent consumption of antibiotics accentuates the relevance of exploring alternative antimicrobial agents. The current investigation highlights the synergistic effect of various Pakistani honeys combined with Nigella sativa, or black seeds, in combating foodborne pathogens, while also assessing the minimum inhibitory concentration (MIC) of these combined extracts.

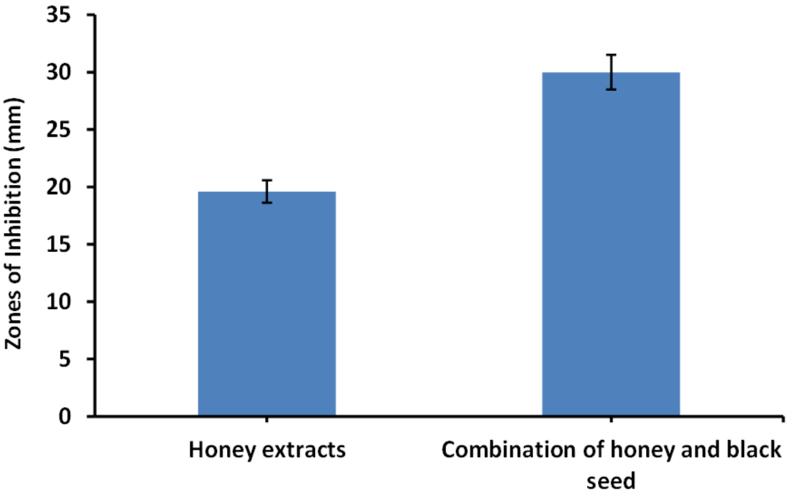


*Figure 1. Graphical representation of the antibacterial activity of honey extract alone and in combination with black seeds extract.*

Ziziphus honey, in particular, demonstrated notable antibacterial activity with an inhibition zone of 19.6 mm against Salmonella typhi, underscoring its potential as a robust antimicrobial agent. This finding aligns with prior research which identified Ziziphus honey as effective against a spectrum of pathogens. Sidr honey also exhibited significant inhibitory properties, particularly in higher concentrations against Escherichia coli (13). The varying levels of antibacterial activity among the honey samples can be attributed to the distinct chemical compositions of each variant, influencing their efficacy against specific pathogens. Gram-negative bacteria often exhibit higher resistance, likely due to their complex cell wall structures, which poses a challenge to the antibacterial compounds in honey.

The study also focused on the antibacterial properties of black seed extracts. The extracts displayed substantial activity against Escherichia coli and Salmonella typhi, with inhibition zones measuring up to 18.0 mm and 15.0 mm, respectively. This efficacy mirrors findings from other studies where Nigella sativa demonstrated significant antibacterial effects, further enhanced when combined with other substances such as turmeric (16). The ethanolic extract of black seeds also showed promising results against Bacillus cereus (17), indicating a broad spectrum of activity that may be beneficial in food preservation.

The synergistic potential of combining honey and black seed extracts was particularly noteworthy. The mixture of Ziziphus honey and black seeds produced the largest inhibition zones against all tested pathogens, illustrating the enhanced antimicrobial effect achieved through this combination. Such synergistic effects not only improve the efficacy of the extracts but also reduce the required dosages, potentially decreasing the likelihood of adverse effects and the development of resistance.

In terms of MIC, the combination of Ziziphus honey and black seeds achieved values as low as 3.12 mg/ml against both Escherichia coli and Staphylococcus aureus, and 6.25 mg/ml against Salmonella typhi. These results are encouraging compared to previous studies where higher concentrations were necessary to achieve similar effects (21, 22). The active compounds in honey and black seeds, including phenolic acids, hydrogen peroxide, and flavonoids, contribute to this efficacy.

Despite these promising findings, the study is not without limitations. The variation in the antibacterial activity of different honey types suggests that not all honey is equally effective against all pathogens. Moreover, the specific conditions under which the extracts are most effective require further elucidation to optimize their use in practical applications. Additionally, while the study supports the use of these natural products as potential alternatives to conventional antibiotics, further research is necessary to fully understand their mechanisms of action and to confirm their safety and effectiveness in clinical settings.

The excessive use of antibiotics and chemical preservatives poses significant health risks, including the emergence of multidrug-resistant organisms and ecological imbalances. The findings from this study advocate for the exploration of natural antimicrobial

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agents and their synergisms, which could provide viable alternatives to address these challenges and contribute to safer food preservation methods.

## CONCLUSION

The study demonstrated that extracts of honey and black seeds, particularly when combined, exhibit significant antibacterial efficacy against foodborne pathogens. This is especially true for Ziziphus honey, which showed enhanced activity in synergy with black seeds. These natural extracts present a viable alternative to traditional antibiotics, potentially mitigating the adverse effects associated with their use and helping to curb antibiotic resistance. Further research is warranted to explore the broader application of these natural antimicrobials in replacing antibiotics and their positive implications on human gut microflora. Continued investigation into novel antimicrobial agents is crucial for addressing the challenges posed by multidrug-resistant organisms.

## REFERENCES

1. Bintsis T. Foodborne pathogens. AIMS microbiology. 2017;3(3):529.
2. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. Pharmacy and therapeutics. 2015;40(4):277.
3. Javadi SMR, Hashemi M, Mohammadi Y, MamMohammadi A, Sharifi A, Makarchian HR. Synergistic effect of honey and Nigella sativa on wound healing in rats. Acta cirurgica brasileira. 2018;33:518-23.
4. Serwecińska L. Antimicrobials and antibiotic-resistant bacteria: a risk to the environment and to public health. Water. 2020;12(12):3313.
5. Farid N, Waheed A, Motwani S. Synthetic and natural antimicrobials as a control against food borne pathogens: A review. Heliyon. 2023.
6. Mandal MD, Mandal S. Honey: its medicinal property and antibacterial activity. Asian Pacific journal of tropical biomedicine. 2011;1(2):154-60.
7. Almasaudi S. The antibacterial activities of honey. Saudi journal of biological sciences. 2021;28(4):2188-96.
8. Hussain MB, Hannan A, Akhtar N, Fayyaz GQ, Imran M, Saleem S, et al. Evaluation of the antibacterial activity of selected Pakistani honeys against multi-drug resistant Salmonella typhi. BMC complementary and alternative medicine. 2015;15:1-9.
9. Forouzanfar F, Bazzaz BSF, Hosseinzadeh H. Black cumin (Nigella sativa) and its constituent (thymoquinone): a review on antimicrobial effects. Iranian journal of basic medical sciences. 2014;17(12):929.
10. Parveen A, Zahiruddin S, Agarwal N, Siddiqui MA, Ansari SH, Ahmad S. Modulating effects of the synergistic combination of extracts of herbal drugs on cyclophosphamide-induced immunosuppressed mice. Saudi Journal of Biological Sciences. 2021;28(11):6178-90.
11. Combarros-Fuertes P, Fresno JM, Estevinho MM, Sousa-Pimenta M, Tornadijo ME, Estevinho LM. Honey: another alternative in the fight against antibiotic-resistant bacteria? Antibiotics. 2020;9(11):774.
12. Pancu DF, Scurtu A, Macasoi IG, Marti D, Mioc M, Soica C, et al. Antibiotics: conventional therapy and natural compounds with antibacterial activity—a pharmaco-toxicological screening. Antibiotics. 2021;10(4):401.
13. Ekhtelat M, Ravaji K, Parvari M. Effect of Iranian Ziziphus honey on growth of some foodborne pathogens. Journal of natural science, biology, and medicine. 2016;7(1):54.
14. Owayss AA, Elbanna K, Iqbal J, Abulreesh HH, Organji SR, Raweh HS, et al. In vitro antimicrobial activities of Saudi honeys originating from Ziziphus spina-christi L. and Acacia gerrardii Benth. trees. Food science & nutrition. 2020;8(1):390-401.
15. Foroughi A, Pournaghi P, Tahvilian R, Zangeneh MM, Zangeneh A, Moradi R. Ethnomedicinal plants: Study on the chemical composition and antibacterial activity of the Nigella sativa (Black seed) oil's. International Journal of Pharmaceutical and Clinical Research. 2016;8(11):1528-32.
16. Chowdhury SP, Banik A, Aurin SA, Saima S. Comparison of individual and synergistic antimicrobial activity of common spices against certain infectious pathogen in Bangladesh. American Journal of Plant Sciences. 2019;10(9):1599-611.
17. Khan AR, Kour K. Wide spectrum antibacterial activity of Nigella sativa L. seeds. IOSR Journal of Pharmacy. 2016;7(6):12-6.
18. Kota CS, Spoorthy Paladi SP. Evaluation of antibacterial activity of Syzygium aromaticum, Laurus nobilis and Cuminum cyminum extracts and their combination. 2013.
19. Baljeet S, Simmy G, Ritika Y, Roshanlal Y. Antimicrobial activity of individual and combined extracts of selected spices against some pathogenic and food spoilage microorganisms. International Food Research Journal. 2015;22(6):2594.
20. Banik A, Abony M, Zerin T, Datta S. Antibacterial activity of Allium sativum, Syzygium aromaticum, and Cinnamomum zeylanicum against food borne pathogens in vitro. IOSR Journal of Pharmacy and Biological Sciences. 2018;13:68-73.

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21. Halawani EM, Shohayeb MM. Shaoka and Sidr honeys surpass in their antibacterial activity local and imported honeys available in Saudi markets against pathogenic and food spoilage bacteria. Aust J Basic Appl Sci. 2011;5(4):187-91.

22. Zam W, Harfouch R, Bittar S, Sayegh M. Antibacterial activity of various Syrian honey types against Pseudomonas aueruginosa. Research Journal of Pharmacognosy and Phytochemistry. 2017;9(2):73-6.