Original Research Article

Antibacterial efficacy of black seed honey in combination with penicillin and amoxiclav against gram-positive bacteria

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ABSTRACT

Background: The emergence of antimicrobial resistance possesses a great threat for the existence of mankind. Antibiotics like penicillin and amoxiclav are at the brink of losing their efficacy entirely in exposure to resistant bacteria. Thus, the present study was aimed to find out the antibacterial efficacy of black seed honey as an alternative natural source which can act independently and boost the efficacy of standard drugs alongside.

Methods: Penicillin, amoxiclav and black seed honey were first individually trailed against four gram-positive bacteria - Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis and Micrococcus luteus. Afterwards, penicillin and amoxiclav were used in combination with honey and compared the synergistic effects with their individual efficacy. Zones of inhibition from well diffusion method, percentage inhibition, minimum inhibitory and bactericidal concentrations by microdilution method were determined in the present study.

Results: Black seed honey alone demonstrated great inhibitory potential against S. aureus (9.7 mm), S. epidermidis (9.9 mm) and M. luteus (9.3 mm) in well diffusion method. Moreover, its combination with amoxiclav showed synergistic effect against all bacteria except S. epidermidis. However, its conjugation with penicillin was not able to produce any synergism as exhibited by zones of inhibition. The lowest concentration (1.56%) of honey applied individually or in combination in microdilution method found highly effective which established an inverse dose dependent relationship with efficacy.

Conclusions: From the data it can be concluded that the black seed honey is a highly potent natural agent which can be utilized in antimicrobial therapy. However, further investigation is recommended to identify the responsible compound for such activity.

Keywords: Black seed honey, Well diffusion, Percentage inhibition, Minimum inhibitory, Minimum bactericidal concentration

INTRODUCTION

Nigella sativa (known as black seed), is an annual flowering herb of Ranunculaceae family and widely found in Europe, Middle East and Western Asia with its many local names, e.g. black cumin (English), black caraway seeds (USA), shonaiz (Persian) and kalajira (Bengali).¹ Honey derived from black seed flower is considered one of the oldest sweeter since ancient time and famous as a natural agent in the treatment of wounds, dyspepsia,
peptic ulcer, gastritis and liver disease. In general, honey has 1% to 5% water and 95% to 99% sugar containing fructose, glucose, maltose, sucrose and isomaltose. Even though the presence of minerals is very low, the honeydew has higher content of minerals than blossom honey, such as calcium, copper, iron, manganese, phosphorus and potassium as the most abundant. Honey carries the combination of organic acids (gluconic acid), phenolic acid, flavonoids, enzymes (invertase, diastase, glucose oxidase) and vitamins (thiamine B, B2 complex and B6) which facilitates collagen structure and fibroblast deposition during wound healing. Many researchers have found its pharmacological properties (anti-inflammatory, anti-oxidant, anti-cancer, anti-parasitic, immune stimulation etc.) by investigating its active components through in vivo and in vitro studies. Apart from that, honey has found to have antimicrobial action against a broad spectrum of pathogenic bacteria and fungi in many laboratory studies. Moreover, it has reported that honey has an inhibitory effect to about 60 species of bacteria, which includes aerobes, anaerobes, gram-positives and gram-negatives. Some studies suggested that the osmotic effect, acidity, hydrogen peroxide and phytochemical factors optimize the antimicrobial activity in honey.

As the worldwide expanded progression of antimicrobial agents brought the threat of antimicrobial resistance due to its misuse and overuse, the easiest way to resist is by facilitating the action of standard drugs through some natural agent. Honey derived from the black seed flower has been reported traditionally effective as a natural antimicrobial agent, but its catalytic potential for standard antibiotics has not yet been evaluated through scientific investigation. Thus, the present study was conducted with an aim to evaluate the efficacy of black seed honey (BSH), penicillin, amoxicillin-clavulanic acid and the combinations to compare with.

**METHODS**

**Sample collection and preparation**

To conduct the study, black seed honey was collected from Hariatpur Sadar Upazila (23°12.5'N 90°21'E) of Shariatpur district of Bangladesh in February 2019. To get the samples, at first a natural hive was disrupted to gather around 2 kg of black seed honey. After that honey was sieved through 0.5 mm mesh and placed at 25±2°C temperature in an impermeable glass container in order to make the sample contamination and moisture free.

**Antimicrobial properties**

**Bacterial strains collection**

Four gram-positive bacterial strains (Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis and Micrococcus luteus) were collected (isolated from feces, cough, nasal mucosa and urine culture respectively) as a token of gift from Center for Medical Biotechnology, Institute of Public Health, Bangladesh.

**Inoculums preparation**

After collecting the four strain samples, they were kept in the nutrient broth tubes for preserving and added 5 ml of sterile saline water to incubate overnight at 37±1°C. For proper growth, samples were sub-cultured in Mueller-Hilton agar (MHA) plates. The spectrophotometer was used for adjusting the absorbance at 580 nm and diluted to have viable cell count of 10⁷ CFU/ml.

**Antimicrobial susceptibility test**

To determine antibiotic susceptibility in the study, the well diffusion strategy was conducted. At first, the sample strains were individually streaked through a sterile cotton swab over the prepared 90 mm MHA plate. The plates were set apart into five equivalent zones. A well of 6 mm was cut from each zone by using a sterile cork borer. After that, 20 μl of test agents were added into the respective wells. During the test, a 10 μl of 10 μg or 10 μl phenoxyethylpenicillin (Sanofi Aventis BD Ltd.) and amoxicillin/clavulanic acid (Sanofi Aventis BD Ltd.) was considered as positive controls and sterile distilled water was considered as negative control. The plates showed diameter in mm of zones of inhibition after its incubation at 37±1°C for a day.

**Minimum inhibitory concentration**

Minimum inhibitory concentration (MIC) was determined through the method described by Patton et al. with slight modifications. A two-fold serial dilution was performed from the stock of raw honey to produce 50%, 25%, 12.5%, 6.25%, 3.12% and 1.56% (v/v) concentrations in a 96-well microplate. At first, phenoxyethylpenicillin, amoxicillin/clavulanic acid and honey at different concentrations were applied individually to assess the individual efficacy against the resistant bacteria. Afterwards, these concentrations of diluted honey were evaluated in combination with the standards where each well constituted 200 μl of nutrient broth, 10 μl of bacterial suspension, 20 μl of honey samples and 10 μl of standard antibiotics. The negative control was devoid of any honey samples and the antibiotics. Optical density of the wells was measured through Biobase-EL10A ELISA Reader (China) to record the initial value (TO). Another reading was taken after a 24 h incubation at 37±1°C to record the value of T24. Percentage inhibition was calculated from the below formula:

\[
\text{Percentage inhibition} = \frac{1 - (\text{OD test/OD control}) \times 100}{100}
\]

MIC was determined from the visual inspection of the well where no turbidity was reported at the lowest applied concentrations.
Minimum bactericidal concentration

From the 96-well plate prepared for the MIC determination, a 20 μl of suspension was transferred to the freshly prepared agar plates from the wells that exhibited MIC. The plates were incubated for 24h at 37±1°C and observe for any bacterial growth. The agar plates which resulted with no bacterial growth is considered the concentration for minimum bactericidal effect.

Statistical analysis

To perform the statistical analysis of the obtained data, one-way analysis of variance (ANOVA) was been used and p<0.05 was considered statistically significant. The collected data for zones of inhibition were measured as mean±standard deviation.

RESULTS

Figure 1 demonstrated that the combination of amoxiclav and honey at its two lower concentrations found to be synergistically effective against B. subtilis (10.2 and 11.1 mm), S. aureus (10.9 and 10.2 mm) and M. luteus (11.2 and 10.6 mm) in respect of zone of inhibition. Alongside, honey alone showed great inhibitory potential against the bacteria specially against S. epidermidis (9.9 mm).

From the calculation of percentage inhibition (Figure 2a-d), it can be observed that the combination of amoxiclav and honey almost inhibited (up to 100%) all bacterial growths. BSH 1.56% alone was found highly effective against S. aureus (97.3%) and S. epidermidis (99.4%). However, at the same concentration the combination of penicillin and honey found less effective than that of honey alone and of the other combination against S. aureus (83.1%) and M. luteus (81.7%). The applied samples established an inverse relationship which exhibited higher potential with lower gradient concentrations.

Black seed honey alone exhibited its inhibitory potential at 1.56% concentration against all four bacteria and was able to kill S. aureus and S. epidermidis with the same concentration (Table 1). Its combination with phenoxybenzylpenicillin found to inhibit S. epidermidis, S. aureus and M. luteus and kill S. aureus at 1.56% concentration of honey. On the other hand, combination of amoxiclav and honey (1.56%) showed inhibitory activity against all species however, bactericidal effect against S. aureus and M. luteus with 3.12% honey.

![Figure 1: Relationship between doses and corresponding zones of inhibition by well diffusion method.](image)

P=Penicillin, A/C=amoxicillin-clavulanic acid, BSH=black seed honey, P+BSH=combination of penicillin and black seed honey, A/C+BSH=combination of amoxicillin-clavulanic acid and black seed honey. Data represents diameter (mm) of zone of inhibition expressed as mean±standard deviation, (n=3); *p<0.05, **p<0.01; Dunnett t-test (two sided) treated one group as control (no antibacterial agent) and compared all other groups against it.
Figure 2 (a-d): Percentage inhibition of bacterial growth by black seed honey.
P=Penicillin, A/C=amoxicillin-clavulanic acid, BSH= black seed honey, P+BSH=combination of penicillin and black seed honey, A/C+BSH=combination of amoxicillin-clavulanic acid and black seed honey. Data represents inhibition of bacterial growth observed in micro-wells expressed as percentage (%), treated one group as control (no antibacterial agent applied) and compared all other groups against it.

Table 1: Determination of MIC and MBC against test bacteria.

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Sample</th>
<th>MIC</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>A/C</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>BSH</td>
<td>BSH 1.56%</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>P + BSH</td>
<td>P 1 μg/μl + BSH 1.56%</td>
<td>P 1 μg/μl + BSH 1.56%</td>
<td>N/A</td>
</tr>
<tr>
<td>A/C + BSH</td>
<td>A/C 1 μg/μl + BSH 3.12%</td>
<td>A/C 1 μg/μl + BSH 1.56%</td>
<td>N/A</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>A/C</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>BSH</td>
<td>BSH 1.56%</td>
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<tr>
<td>P + BSH</td>
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</tr>
<tr>
<td>A/C + BSH</td>
<td>A/C 1 μg/μl + BSH 3.12%</td>
<td>A/C 1 μg/μl + BSH 3.12%</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Continued.
Penicillin and amoxiclav were among the earliest discovered antibiotics known to have broad spectrum of activity against almost any types of bacteria. However, soon after the emergence of bacterial resistance, these potent antibiotics started to lose their efficacy. Penicillin is a beta lactam antibiotic which blocks the cell wall synthesis of bacteria by inhibiting the transpeptidase enzyme. Peptidoglycan, an integral structural component of bacterial cell wall, is interfered by penicillin which prevents the cross-linking during biosynthesis of cell wall. As a result, bacteria become vulnerable to outside environment and molecular pressure.\textsuperscript{13} Phenoxy-methyl penicillin, exhibits bactericidal effect against penicillin-sensitive microorganisms during the stage of active multiplication.\textsuperscript{14} But the beta-lactam ring of phenoxyethylpenicillin is ruptured by the bacterial beta-lactamase enzyme and results in either alteration of target affinity of penicillin or decrease penetration of penicillin towards the target site which can be attributed to the mechanism of penicillin resistance.\textsuperscript{15} On the other hand, amoxicillin is a semisynthetic penicillin, works by the same mechanism of action.\textsuperscript{16} The combination of amoxicillin and clavulanic acid exerts bactericidal effect against a wide range of gram-positive and gram-negative bacteria.\textsuperscript{17} Clavulanic acid as a beta lactam, structurally resembled to penicillin, gets exposed to beta lactamase, thereby, protecting and facilitating the activity of amoxicillin. Clavulanic acid itself is not clinically effective to produce prospective antimicrobial response. However, the hyperproduction of TEM-1 b-lactamase can also cause resistance and it is the most frequent contributor as found so far.\textsuperscript{18} Honey is a natural preservative agent composed of at least 181 compounds including simple sugars, vitamins, proteins and free amino acids, polyphenols, carotenoids, flavonoids, calcium, potassium, phosphorus, iron, niacin, ascorbic acid and minerals and yet many unidentified compounds.\textsuperscript{19,20} Presence of defensins along with its consistent amount of hydrogen peroxide and non-peroxide factors like polyphenols and flavonoids content, high sugar content, osmotic effect, low pH level attribute to the antimicrobial effect of the honey.\textsuperscript{21,22} When used in combination, these factors might have boosted the potency of penicillin and amoxiclav.

The inverse relation of dose and efficacy potentiate another interesting field of investigation. But at this stage of the study it can be attributed to the high concentration factor of honey. At high concentration, it might have acted solely as preservative which inhibited the growth. The antibiotics could not get the room for diffusion due to the high viscosity. However, with dilution, active compounds of the honey started working side-by-side with the antibiotics and thereby, boosted the efficacy.

**CONCLUSION**

The present study concludes that black seed honey not only pose great antibacterial potential alone but also was able to boost the efficacy of phenoxyethylpenicillin and amoxiclav to resist bacterial growth. further study is needed to find out the responsible compounds and similar trials are recommended to evaluate its potential against other microorganisms and in combination with other antibiotics as well.

**ACKNOWLEDGEMENTS**

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**Ethical approval:** Not required

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