Wild honey facilitates antibacterial efficacy of penicillin and amoxicillin–clavulanic acid

Mahi Khan1, Zahirul Islam2, Ayreen S. Chowdhury2, Syed A. Yousuf3, Md. R. Amin4, Md. A. Rayhan5
1Department of Pharmaceutical Sciences, North South University, Dhaka, Bangladesh
2Department of Pharmacy, University of Asia Pacific, Dhaka, Bangladesh
3Department of Anesthesiology, Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh
4Center for Medical Biotechnology, Directorate General of Health Services, Dhaka, Bangladesh
5Department of Pharmacology, Institute for Pharmaceutical Skill Development and Research, Dhaka, Bangladesh

ABSTRACT

Background: Antibacterial efficacy of honey has been well acknowledged from ancient to modern society. Utilizing a wild honey, the present strategy of the study was chosen to address an unfilled demand for finding a complement of penicillin analogs which have already been reported ineffective in many cases.

Methods: In this study, penicillin and amoxiclav were trialed against four clinically resistant bacteria – two Gram positives (Staphylococcus aureus and Streptococcus pyogenes) and two Gram negatives (Klebsiella pneumoniae and Acinetobacter baumannii). Afterward, these bacteria were exposed to different concentrations of a natural wild honey. Finally, the honey was used in conjugation with penicillin and amoxiclav individually. The zone of inhibition, minimum inhibitory, and bactericidal concentrations were observed in this regard.

Results: The wild honey showed a great potential to inhibit the bacterial growth at its lowest dose given (6.25%) both against Staphylococcus (48%) and Streptococcus species (45%) but could not exhibit any bactericidal effect alone. However, it greatly influenced both penicillin (100%) against Acinetobacter and amoxiclav (93%) against Klebsiella species, whereas these two antibiotics could not demonstrate a good antibacterial effect when applied individually against these bacteria.

Conclusion: All findings suggested that wild honey not only possesses an inhibitory effect but also facilitates the action of other agents, thereby generating a scope for research for identifying new potential antibacterial agents.

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Introduction

Antibiotic resistance is a global concern raising the potential threat to individual health and eventually to socio-economic development. The underdeveloped countries are at the most susceptible position having less or no prevention strategies that can effectively control the eminent outbreak. This is mainly due to the irrational use of antibiotics [1,2]. As a consequence, previously useful antibiotics have lost their efficacy, and broader-spectrum antibiotics are being brought into action. As an inherent characteristic, bacteria gradually develop resistance toward applied antibiotics by mutation, which implies the great risk for destroying the last-line agents for controlling the infectious diseases [3]. Once considered a miraculous drug, penicillin now found ineffective against almost all types of infections [4]. Amoxiclav, a highly potent combination drug, is being reported as ineffective in controlling diseases [5]. Where the invention process of new antibiotics is time-consuming, the bacteria are getting resistant to available antibiotics faster than ever. Thus, the crying need of antibiotic invention can only be supplemented by naturally occurring antibacterial agents. Honey is such one.

Honey is a traditional medicine, which has been tested and reported effective for many types of
Ailments [6–8]. Honey is not just a sugar rather a complex combination of enzymes, organic acid, trace materials, and yet unidentified compounds. Among the identified, at least 181 components, including simple sugars, proteins, free amino acids, vitamins, polyphenols, flavonoids, carotenoids, calcium, phosphorus, iron, niacin, minerals, and ascorbic acid, have been confirmed in honey [9,10]. The pharmacological properties exhibited by honey depend largely on its composition; however, the actual composition of honey varies in association with many factors such as the honey bees and angiosperm species, climate, and the process that it undergoes [11]. Due to its high sugar content, honey exhibits antibacterial properties. However, the antibacterial properties are highly complex to describe due to the involvement of multiple compounds and the large variation in the concentrations of these compounds among kinds of honey as well [12]. Bees collect nectar from different flowers and make a hive to store it. Wild honey is considered more effective because of the variety of compounds found in it. Honey contributes to both internal and external healings. Honey has many pharmacological effects, such as antibacterial, antioxidant, anti-inflammatory, and antiallergenic activities, apart from its metabolic activities for the body [13]. Many reports have been found on its pharmacological properties such as antibacterial [14]; however, very few have been focused so far on increasing the efficacy of weak antibiotics by its conjugation. Thus, the present study was aimed to boost the efficacy of penicillin and amoxiclav with the help of wild honey against resistant bacteria.

Methods

Collection and preparation of the sample

In the month of May, approximately 2 kg of honey was collected after breaking a natural hive from a wild tree found at Sunamganj District (25.0715° N, 91.3992° E) of Sylhet Division of Bangladesh. After the collection of honey, it was sieved through a mesh of 0.5 mm for eliminating any kind of coarse particles. It was kept at 25°C ± 2°C temperature in an impermeable glass container to avoid the accumulation of moisture.

Physiochemical properties

Determination of moisture content and total soluble solids (TSS)

A method described by Bogdanov et al. [15] was performed for the determination of the presence of moisture content and TSS in honey [16]. Before using a honey refractometer (Biobase BK-PRN3 China), it was thermoregulated and calibrated. To observe the moisture content and TSS, a droplet of honey is applied to the prism of refractometer, and the values displayed through the lens of the refractometer were noted.

Determination of pH

For measuring pH of the honey, Biobase pH-10S (China) pH meter was used after calibration at pH 4.01 and 7.00 with standard buffer solution. From the honey sample, a 10% (w/v) solution was prepared by the addition of distilled water, and the reading was taken in triplicate [17].

Determination of the optical density (OD)

For measuring OD, a 10% (w/v) honey solution was prepared. Using the Biobase BK-UV1800 UV-VIS spectrophotometer (China) keeping the distilled water as blank, the absorbance was taken at 530 nm. Finally, from the absorbance value, the color of the honey was determined from the color chart set by the United States Department of Agriculture [18,19].

Determination of honey density

Honey density was determined using the following formula, where the mass of the honey was measured from the difference of both empty and filled weights of a 1-ml syringe using an automatic electronic analytical balance Biobase BA2004N (China) [20].

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\text{Density of honey} = \frac{\text{Mass of honey}}{\text{Volume of honey}}
\]

Antimicrobial properties

Collection of bacterial strains

Four bacteria, two Gram-positive (Staphylococcus aureus and Streptococcus pyogenes) and two Gram-negative (Klebsiella pneumoniae and Acinetobacter baumannii) isolates from urine culture were collected as a gift from the bacterial line collection of the Center for Medical Biotechnology, Institute of Public Health, Bangladesh.

Preparation of inoculums

Collected bacterial strains were subcultured overnight at 37°C ± 1°C in Mueller–Hilton agar (MHA) plates and Nutrient Broth (NB) tubes. The bacterial growth was allowed using 5 ml of sterile saline water, and its absorbance was adjusted at 580 nm
and diluted to attain a viable cell count of $10^7$ CFU/ml using spectrophotometer [21].

**Antimicrobial susceptibility test**

The well-diffusion method was applied to perform the antibiotic susceptibility test [22]. Using a sterile cotton swab, each bacterial strain was streaked over the prepared 90-mm MHA plate. Each plate was marked into five equal zones, and from each zone, a 6-mm well was cut using a sterile cork borer. About 20 μl of test agents were poured into the respective wells. Nearly 10 μl of 10 μg/10 μl phenoxymethylpenicillin [Sanofi Aventis (BD) Ltd.] and amoxicillin/clavulanic acid [Sanofi Aventis (BD) Ltd.] were used as positive controls, and sterile distilled water was served as negative control. The plates were then incubated at $37^\circ C \pm 1^\circ C$ for 24 hours and finally observed for the zone of inhibition (diameter in mm). Each assay was repeated in triplicate.

**Minimum inhibitory concentration**

The minimum inhibitory concentration (MIC) was determined using the microdilution technique according to Patton et al. with some minor modifications [23]. A 96-well microplate was used, where two-fold serial dilution was made from the stock honey (100%) to generate the concentrations of 50%, 25%, 12.5%, and 6.25% (v/v) using sterile distilled water. These concentrations were applied in combination with phenoxymethylpenicillin and amoxicillin/clavulanic acid separately in respective wells for comparison. Each antibiotic well contained 200 μl of NB, 10 μl of bacterial suspension, 10 μl of standard antibiotics, whereas the honey well contained 20 μl of honey sample in the place of the antibiotic. For combination wells, both antibiotic (10 μl) and honey (20 μl) were added. Finally, NB was added to make a final volume of 300 μl for each well. The negative control did not contain any test agents or antibiotics. A reading of the absorbance of the microwells was taken through Biobase-EL10A ELISA Reader (China) to consider the initial value ($T_0$). The plates were allowed for incubation at $37^\circ C \pm 1^\circ C$ for 24 hours, and again, the absorbance was taken ($T_{24}$). From the difference, the percentage inhibition was calculated using the following formula:

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

The wells that visually exhibited no turbidity with the least concentration were considered as the MIC.

**Minimum bactericidal concentration**

To determine the minimum bactericidal concentration (MBC), 20 μl suspension from the well that exhibited invisible growth with the lowest concentration was transferred into MHA plates that did not contain any test agents or antibiotics. The plates were then incubated at $37^\circ C \pm 1^\circ C$ for 24 hours and observed for bacterial growth. MBC was taken as the concentration of honey or its combination that did not exhibit any bacterial growth on the freshly inoculated agar plates [24].

**Statistical analysis**

All analyses were performed in triplicate, and the data were expressed as mean ± standard deviation. Differences between the activities of the kinds of honey as measured by the zones of inhibition were analyzed using one-way analysis of variance, and $p<0.05$ was considered to be statistically significant.

**Results**

**Physicochemical properties of honey**

Table 1 shows that the wild honey has a low moisture content. The color intensity indicates light amber color according to the USDA color chart, and the pH value indicates its acidic nature.

**Antimicrobial properties of honey**

After an incubation period of 24 hours, the Petri dishes were visually inspected for the zone of inhibition. The diameters of the resulted zones were measured using a Vernier caliper scale. Figures 1–3 shows the relationship of samples used in different doses with corresponding zones of inhibition.

Figure 1 shows that the two standards such as penicillin and amoxiclav had a negligible inhibitory effect over the tested bacteria. Wild honey exhibited the significant growth inhibition properties at different doses but could not establish any dose-dependent relationship except against...
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**Klebsiella**, in which the lowest concentration of the honey (6.25%, 11.4 mm) found to be more effective than that of higher concentration (100%, 7.0 mm). This inverse relationship was also observed for **Streptococcus** when the honey was conjugated with penicillin (Fig. 2). On the contrary, Figure 2 also shows that penicillin with raw honey (100%) was found highly effective against other three species.

Figure 1. Comparison of antimicrobial efficacy of penicillin and amoxiclav with different concentrations of wild honey by well diffusion method.

P = Penicillin, A/C = Amoxicillin–Clavulanic Acid, WH = Wild honey. Data represent diameter (mm) of the zone of inhibition expressed as mean ± standard deviation (n = 3); *p < 0.05, **p < 0.01, ***p < 0.001; Dunnett’s t-test (two-sided) treated one group as control (no antibacterial agent) and compared all other groups against it.

Figure 2. Comparison of antimicrobial efficacy of penicillin versus penicillin in combination with different concentrations of wild honey by well diffusion method.

P = Penicillin (1 µg/µl), P+WH = Combination of penicillin (1 µg/µl) and wild honey. Data represent diameter (mm) of the zone of inhibition expressed as mean ± standard deviation (n = 3); *p < 0.05, **p < 0.01; Dunnett’s t-test (two-sided) treated one group as control (no antibacterial agent) and compared all other groups against it.
The maximum peak of antibacterial efficacy (A/C+WH 25%, 18.6 mm) was exhibited by honey with amoxiclav against *Klebsiella* species (Fig. 3). The combination was also found to be effective against other species at the highest concentration (100%).

Figure 4 (a and b) shows that wild honey alone exhibited a maximum inhibition at its lowest dose given (6.25%) both against *Staphylococcus* (48%) and *Streptococcus* species (45%). When applied in combination with penicillin against *Staphylococcus* and with amoxiclav against *Streptococcus*, the reductions in efficacy were observed with decreased concentrations. However, the opposite phenomenon was observed with penicillin against *Streptococcus* species.

P = Penicillin (1 µg/µl), A/C = Amoxicillin–clavulanic acid (1 µg/µl), WH = Wild honey, P+WH = Combination of penicillin (1 µg/µl) and wild honey, A/C+WH = Combination of amoxicillin–clavulanic acid (1 µg/µl) and wild honey. Data represent the inhibition of bacterial growth observed in microwells expressed as percentage (%), treated one group as control (no antibacterial agent) and compared all other groups against it.

Amoxiclav with all doses of honey showed a multifold increase in inhibition when applied against *Klebsiella* (Fig. 4c) at decreased concentrations. Penicillin when used with honey 100% showed the maximum inhibition (73%) against *Acinetobacter* species but decreased gradually with serial dilution of honey (Fig. 4d). Both penicillin and amoxiclav were found almost inactive against the resistant strains.

Table 2 shows the MIC and MBC values against the respective bacteria. Against *S. pyogenes* (Gram positive) and *K. pneumoniae* (Gram negative), the lowest concentration of honey (6.25%) exhibited great synergism with penicillin and amoxiclav, respectively. Penicillin, amoxiclav, and wild honey alone found to have no bacteriostatic or bactericidal effect against the test bacteria.

**Discussion**

Physicochemical properties represent the nature and quality of the honey. The low moisture content of this wild honey indicated that there was a lesser chance for bacteria to undergo fermentation process which leads to degradation of the honey [25]. As a measure of dissolved sugar in honey, more than 80% of TSS signified a high grade of honey and considered highly stable on storage [26]. Acidic nature implied the possibilities of this honey to have high organic or amino acid contents [27].

Penicillin inhibits the transpeptidase enzyme that catalyzes the final step of cell wall biosynthesis and the cross-linking of peptidoglycan and,
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thereby, kills the susceptible bacteria. Devoid a cell wall, the bacterial cell becomes exposed to outside water and molecular pressures, and eventually, the bacteria die [28]. As a broad-spectrum beta-lactam antibiotic having bactericidal effect against Gram-positive and Gram-negative bacteria, phenoxymethylpenicillin has a mode of action similar to that of benzylpenicillin. However, it is weakened by penicillinase and other beta-lactamases [29,30]. The resistance to phenoxymethylpenicillin is supposed to act by the destruction of the beta-lactam ring by a beta-lactamase, altered affinity of penicillin for target, or decreased penetration of the antibiotic to reach the target site [31].
Another beta-lactam antibiotic, amoxicillin (a semisynthetic penicillin) inhibits one or more penicillin-binding proteins in the pathway of bacterial peptidoglycan biosynthesis (an integral structural component of the bacterial cell wall). Such inhibition leads to the vulnerability of the cell wall, which leads to cell lysis and death [32]. Amoxicillin is susceptible to degrade by beta-lactamases produced by resistant bacteria, and thus, it is not considered a therapeutic choice against organisms which produce these enzymes [33]. Clavulanic acid, a structural beta-lactam analog of penicillin, inactivates some beta-lactamase enzymes and therefore prevents inactivation of amoxicillin [34]. Clavulanic acid alone does not exert a clinically useful antibacterial effect. Resistance to amoxiclav is mediated by both clavulanate-resistant enzymes and hyperproduction of TEM-1 b-lactamase; however, hyperproduction mechanism has been by far found as the most frequent contributor [35].

As a prophetic medicine, honey has been recommended not only as a nutrient but also as a medicine and preservative [36,37]. Honey exerts antibacterial properties due to the presence of defensins as well as its consistent amount of hydrogen peroxide and nonperoxide factors, such as flavonoid and polyphenol content, low pH level, and osmotic effect (due to high sugar content) [38,39]. Moreover, methylglyoxal and the antimicrobial peptide bee defensin-1 were found to contribute to the antibacterial properties of honey [9]. The addition of honey to antibiotics increased the zone of inhibition and inhibition in broth but for certain combinations was unable to show the effect in MIC/MBC tests as only the wells those exhibited no visible turbidity were considered for further MIC/MBC observation. A lower concentration of honey found to have more effect when used alone and with penicillin (against Streptococcus and Klebsiella). The inverse relation of dose and efficacy potentiates another interesting field of investigation. However, 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 a preservative which inhibited the growth. The antibiotics could not get the room for diffusion due to the high viscosity of sugar. However, with dilution, active compounds of the honey started working side-by-side with the antibiotics and, thereby, boosted the efficacy. Further investigation is necessary to identify the responsible compounds by GC-MS analysis, and the mechanism of action can be drawn through transmission electron microscopy as performed by Abdel-Shafi et al. [40].

**Conclusion**

Bacterial resistance against penicillin and amoxiclav has been a prime concern for the health professionals and thus for the research community as well. Many attempts have already been taken to rejuvenate these two antibiotics by structural modification through semisynthesis which was costly and time consuming. In this study, the simple addition of the wild honey to these antibiotics demonstrated a significant synergism rather than their individual application. From the obtained result, it can be concluded that wild natural honey has a great potential to act as a natural antibacterial agent and can be used along with antibiotics to bring these back into their action.

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**Conflict of interest**

All authors agreed on the article before submission and had no conflict of interests.

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