PharmacologyOnLine

Archives • 2021 • vol.2 • 1365-1373

EVALUATION OF ANTIBACTERIAL ACTIVITY OF MUSTARD HONEY AND ITS POTENTIAL TO BOOST THE EFFICACY OF PENICILLIN AND AMOXICLAV AGAINST RESISTANT BACTERIA

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Abstract

The aim of the study was to evaluate the efficacy of mustard honey as a natural antibacterial agent as well as its potential to boost penicillin and amoxiclav in action.

Two gram-positive and gram-negative resistant bacterial strains were examined for their sensitivity towards penicillin, amoxiclav and mustard honey individually and finally towards the honey-antibiotic combinations in different doses. Zone of inhibition, minimum inhibitory concentration and minimum bactericidal concentration were observed.

Mustard honey was able to produce moderate inhibitory action against all strains, maximum against S. aureus (59%) at 6.25% concentration. Whereas the standards could not exhibit such response. Moreover, the honey synergistically enhanced the efficacy of the penicillin and amoxiclav when applied in combination, maximum against *K. pneumoniea* (92% and 98% respectively).

The study concludes that the mustard honey possesses wide-spectrum antibacterial activity and also can act as a natural catalytic agent for the tested antibiotics to give synergistic effects.

Key words: *Mustard Honey, Zone of Inhibition; MIC; MBC*

Introduction

Bacterial resistance to antibiotics though has been a recognized reality almost since the dawn of the antibiotic epoch, from the past two decades it has been in very alarming position. Moreover, this intensifying evolution of resistance tied with a lessened antibiotic pipeline has led the health professionals to claim that a post-antibiotic era is eminent [1]. Penicillin and Amoxiclav once considered miraculously potent antibiotics, now being reported ineffective against commonly occurring bacteria. The structural modification of the molecules is under research however, is costly and time-consuming. This arises the need for alternative and complementary medicine which is yet unfilled.

From the ancient times, honey had been found reported as a folk medicine for its antibacterial efficacy [2]. Study showed that other than its function as natural preservative, it was found potent for abdominal abnormalities, burns, sores wound healing etc. [3]. Apart from its high sugar content, it is enriched with enzymes, organic acid, trace materials, proteins, free amino acids, vitamins, polyphenols, flavonoids, carotenoids, calcium, phos¬phorus, iron, niacin, minerals, and ascorbic acid among the 181 identified compounds [4,5]. Honey derived from Mustard (*Brassica nigra*) has been reported to have antibacterial, antioxidant, detoxifying potential for which it is widely used as a traditional medicine [6,7]. Many studies already undergone over these claims but very few approached to utilize the honey to increase the efficacy of weak antibiotics by combination. Thus, the present study was designed to evaluate the efficacy of mustard honey against resistant bacteria as well as to assess its potential for strengthening penicillin and amoxicillin-clavulanic acid by conjugation.

Methods

Collection and preparation of the Sample

In the month of February, roughly 2kg of Mustard (Brassica nigra) honey (randomly denoted as BNH) was collected after breaking a cultivated hive in the mustard field at Basila upazila (24°13′N 90°3′E) of Tangail District of Bangladesh. After collection, it was sieved through a 0.5 mm mesh for eliminating any sort of coarse particles. It was kept at 25±2°C temperature in an impermeable glass container to avoid accumulation of moisture on the surface of the honey. From the mother stock of the honey (100%); 50%, 25%, 12.5% and 6.25% (v/v) concentrations were prepared by two-fold serial dilution using sterile distilled water [8].

Antimicrobial Properties

Collection of Bacterial Strains

Two gram-positive (Staphylococcus aureus and Streptococcus pyogenes) and two gram-negative (Klebsiella pneumoniae and Acinetobacter baumannii) bacterial isolates from urine cultures were collected from Center for Medical Biotechnology, Institute of Public Health, Bangladesh. The standard strain of *Staphylococcus aureus* (ATCC 6538), *Streptococcus pyogenes* (ATCC 19615), *Klebsiella pneumoniae* (ATCC 13883) and *Acinetobacter baumannii* (ATCC 19606) were obtained and used as references.

Preparation of Inoculums

From the stock of collected bacterial strains, subcultures were prepared in Mueller-Hilton Agar (MHA) plates and Nutrient Broth (NB) tubes by an overnight incubation at 37±1°C. The bacterial development was permitted using 5 ml of clean saline water, its absorbance was adjusted at 580 nm and diluted to attain viable cell count of 107 CFU/ml using spectrophotometer [9].

Antimicrobial Susceptibility Test

The well diffusion method was carried out to determine the susceptibility of the microorganism. Freshly prepared 90 mm MHA plates were streaked with the bacterial strains using sterile cotton swabs where the plates were marked into five equal zones on the bottom using permanent ink markers. Each zone was punched with a cork borer to cut a 6 mm well in which 20µl of test agents were poured respectively. In wells which served for positive control, about 20µl of 1 µg/µl phenoxymethylpenicillin (Sanofi Aventis (BD) Ltd.) and Amoxicillin/Clavulanic Acid (Sanofi Aventis (BD) Ltd.) were applied respectively whereas sterile distilled water assisted as negative control. After a

24h incubation at 37±1°C, the diameter (mm) of zones of inhibition were measured with a Vernier Calipers [10]. The test was repeated for three times.

Minimum Inhibitory Concentration

Micro-dilution technique was adopted to determine the minimum inhibitory concentration (MIC) of the test agents [11]. The respective wells of a 96-well microplate were first filled with 200 µl of NB. Then each well was provided with 10 µl of respective bacterial suspension. Afterwards, the wells for positive controls received 10 µl of standard antibiotics, for test agent received 20 µl of honey's respective concentrations whereas for combinations received both. The wells for negative control received no antibiotics or test agents rather filled with same volume of NB. Finally, the wells were made up to 300 µl by addition of NB. Biobase-EL10A ELISA Reader (China) was used to take the initial absorbance (T0) and the final absorbance (T24) after an incubation period of 24h at 37°C ± 1°C, difference of which was utilized for the calculation of percentage inhibition:

Percentage inhibition = 1 – (OD test/OD control) × 100

MIC was determined from the observation with the least concentration that was able to exhibit no visual turbidity.

Minimum Bactericidal Concentration

Minimum bactericidal concentration (MBC) was extracted from the MIC. The wells those exhibited as MIC were transferred (20µl suspension) to freshly prepared MHA plates without addition of any test agents or antibiotic. After incubation (at 37±1°C for 24h), the plates were observed for bacterial growth. MBC was determined as the least concentration of test agent, antibiotics or combinations devoid of any bacterial development in the incubated plates [12].

Statistical Analysis

All experiments were performed three times and data was expressed as mean ± standard deviation. Contrasts between the activities of the honeys as measured by the zones of inhibition were analyzed by using one-way analysis of variance (ANOVA) and $P < 0.05$ was considered statistically significant.

Results

As being weak antibiotics against resistant bacteria, penicillin and amoxiclav was not able to produce significant zones of inhibition (Figure 1). However, as a natural agent mustard honey exhibited inhibitory properties by displaying greater zone diameters. Among the different concentrations, the lower doses exhibited higher efficacy in terms of inhibition. When applied in combination with the antibiotics, the honey was found with potential synergistic effects. Maximum efficacy was observed by the combination of amoxiclav with lowest applied dose of honey (6.25%) against *Klebsiella* (19.2 mm). Whereas penicillin with maximum dose of honey (100%) found effective against *Klebsiella* (14.8 mm) and *Acinetobacter* (14.6 mm).

Like the outcomes of susceptibility test penicillin and amoxiclav were found ineffective to reduce the turbidity produced by the bacterial growth (Figure 2a-2d). Maximum efficacy demonstrated by penicillin and amoxiclav by their individual application were against *klebsiella* respectively as 14% and 21% of inhibition. At the dose of 6.25%, mustard honey alone was able to produce 59% inhibition against *Staphylococcus* Whereas in combination it boosted the responses of the standard antibiotics up to 81% (amoxiclav). Most enhanced bactericidal activity was detected by the honey (6.25%) in combination with amoxiclav (98%) against *Klebsiella*. Similar response was observed against *Acinetobacter* growth as well. While in the other growth plate of *Streptococcus* incubation, 12.5% honey was found more effective than the other concentrations.

Table 1 showed that the individual application of penicillin, amoxicillin-clavulanic acid and the mustard honey could not result in a specific concentration from the applied doses to identify the MIC and MBC against the tested organisms. But the combinations were found effective to draw the points at which these can be attributed to MIC and MBC. In most of the cases, lower concentrations proved more effective than higher concentrations. However, at any concentration the combinations could not demonstrate bactericidal effect against *S. pyogenes*.

Discussion

Biological activities of honey differ based on the inherent physical properties and chemical constituents of the honey. Both of these natures of honey vary depending on the floral origin, geographical origin, humidity, temperature, climatic, and environment conditions from which the bees collect the nectar [13]. From the previous study, it has been reported that the honey possesses an acidic pH, water white color according to USDA gradation with a mild moisture content which implies to its high organic or amino acid contents as well as lesser chances for degradation [14]. The findings showed that the honey produced moderate inhibitory responses to the test organisms when applied alone in different concentrations.

As a cell wall synthesis inhibitor, a molecule of penicillin acts while a bacterium actively synthesizes its cell wall. The drug mimics the terminal D-Alanine-D-Alanine structure of the amino acid chain which is associated with NAM (N-Acetyl muramic acid) unit of the peptidoglycan chain [15]. Basically, it competitively inhibits the active site of the transpeptidase enzyme for halting the synthesis process of gram-positive bacteria. The resistance to penicillin exhibited by any bacteria is mediated by destruction of the beta-lactam ring by bacterial beta-lactamase, altered affinity towards penicillin binding site, or decreased penetration of the antibiotic to reach the target site [16]. Similarly, amoxicillin inhibits the penicillin-binding proteins (PBPs) required in the pathway of bacterial peptidoglycan biosynthesis and therefore, is susceptible to degrade by beta-lactamases produced by resistant bacteria [17,18]. Clavulanic acid, a structural beta-lactam analogue of penicillin, inactivates some beta-lactamase enzymes therefore prevents inactivation of amoxicillin [19]. Resistance to amoxiclav is mediated by both clavulanate-resistant enzymes and hyperproduction of TEM-1 b-lactamase though hyperproduction mechanism is considered the most frequent contributor [20]. On the other hand, cell wall of gram-negative bacteria is surrounded and protected by another sheath celled outer membrane which resist the drug to penetrate the periplasmic space to exert its action [21]. That is why, cell wall inhibitors like penicillin and amoxiclav are usually found ineffective against gram-negative bacteria. The present study showed that when these drugs were conjugated with honey against gram-negative bacteria, the honey was hypothesized firstly to break the outer membrane or make it permeable for penicillin; secondly to act side-by-side with its inherent constituents to inhibit the bacteria by same or other mode of inhibitory action. However, the responsible components as well as the mode of action for such hypothesis are yet to be investigated.

The antibacterial properties of honey can be attributed to defensins and hydrogen peroxide and non-peroxide factors like flavonoids and polyphenols content, low pH level, osmotic effect etc. Moreover, the contribution of methylglyoxal and peptide bee defensin-1 have also been reported [22,23]. The finding articulated that in most of the cases, lower concentration of honey proved in higher efficacy which is another interesting field of investigation. Overall, at this stage of study, it can be hypothesized that at high concentration, the honey could act as a preservative due to high sugar content where the antibiotics could not diffuse to exhibit their action. But in diluted concentration, not only the sugar but also other component acts parallelly with the antibiotics to give synergistic effects.

Conclusion

In search for a wide-spectrum natural antibacterial agent which can act both against gram-positive and gram-negative bacteria, mustard honey proved to be a prominent one. The current study provides the scientific evidence that mustard honey Not just in its sole application, rather in combination with the standard antibiotics, it demonstrated synergistic potential against resistant bacteria. However, to establish it as a complementary and alternative drug, biotechnological approaches should be adopted to isolate the biologically active components, characterize and produce in massive quantities.

Acknowledgments

The present study was supported and carried out in the Pharmacology lab of Institute for

Pharmaceutical Skill Development and Research, Bangladesh. Authors are grateful to the institution for providing such opportunity to contribute to health science. Authors are also grateful to Center for Medical Biotechnology (CMBT) under MIS, DGHS for providing the bacterial strains used in this study.

Statement of Ethics

The paper is exempt from ethical committee approval.

Conflict of Interest

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

Funding Sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author Contributions

This work was carried out in collaboration between all authors. Authors Mohammad Mustakim Billah designed, coordinated and supervised the project and also performed the statistical analysis. Ayreen Sonia Chowdhury performed in vitro experiments and participated in acquisition of data. MD. Saqline Mostaq analyzed the data. Nelson Halder drafted the manuscript and Md. Ali Imam Razu critically revised the manuscript. Md. Razibul Habib provided technical support and conceptualized the experiments. All authors read and approved the final manuscript.

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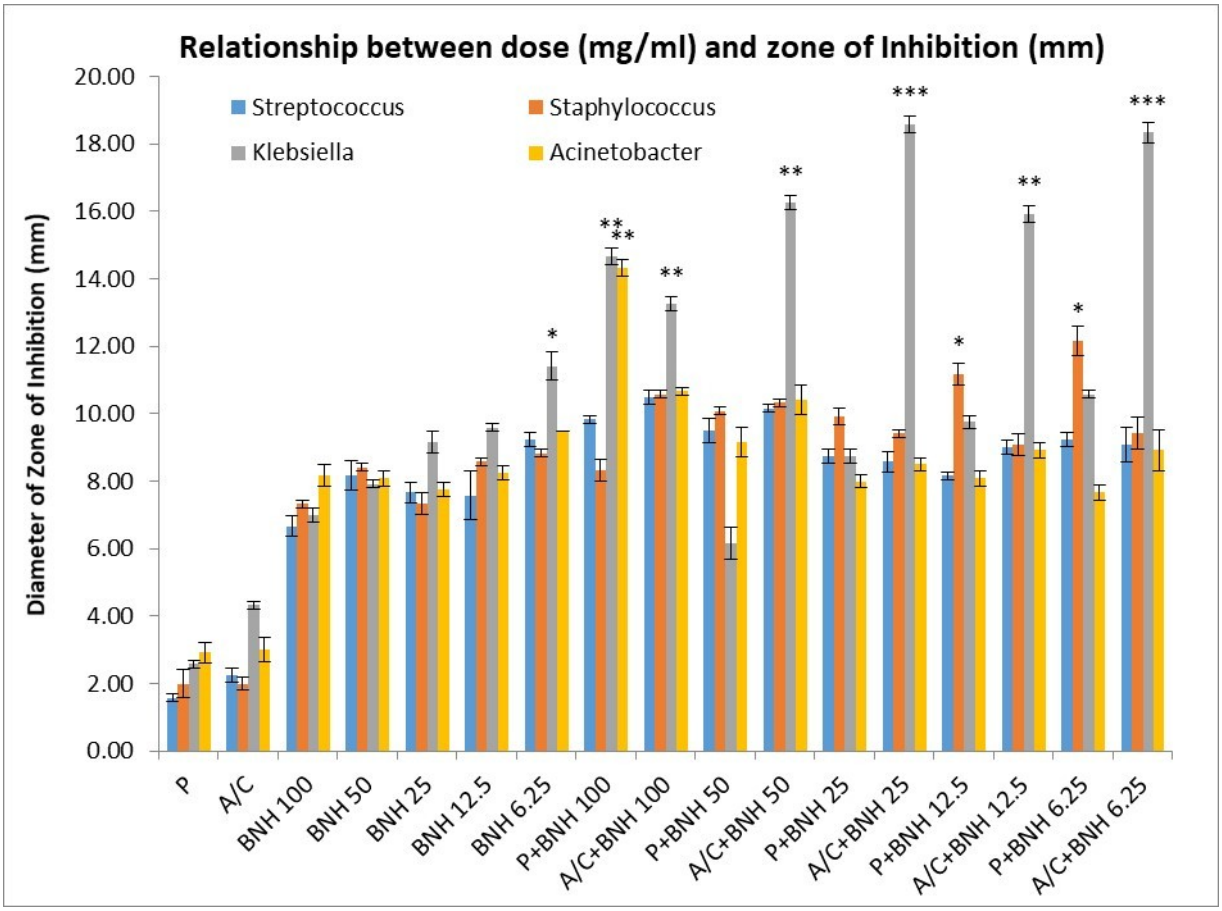

Figure 1: Relationship between doses and corresponding zones of inhibition by well diffusion method.

P=Penicillin, A/C=Amoxicillin-Clavulanic Acid, BNH=Mustard Honey. Data represents diameter (mm) of zone of inhibition expressed as mean ± standard deviation, (n = 3); *p < 0.05, **p < 0.01, ***p < 0.001; Dunnett t-test (two sided) treated one group as control (no antibacterial agent) and compared all other groups against it.

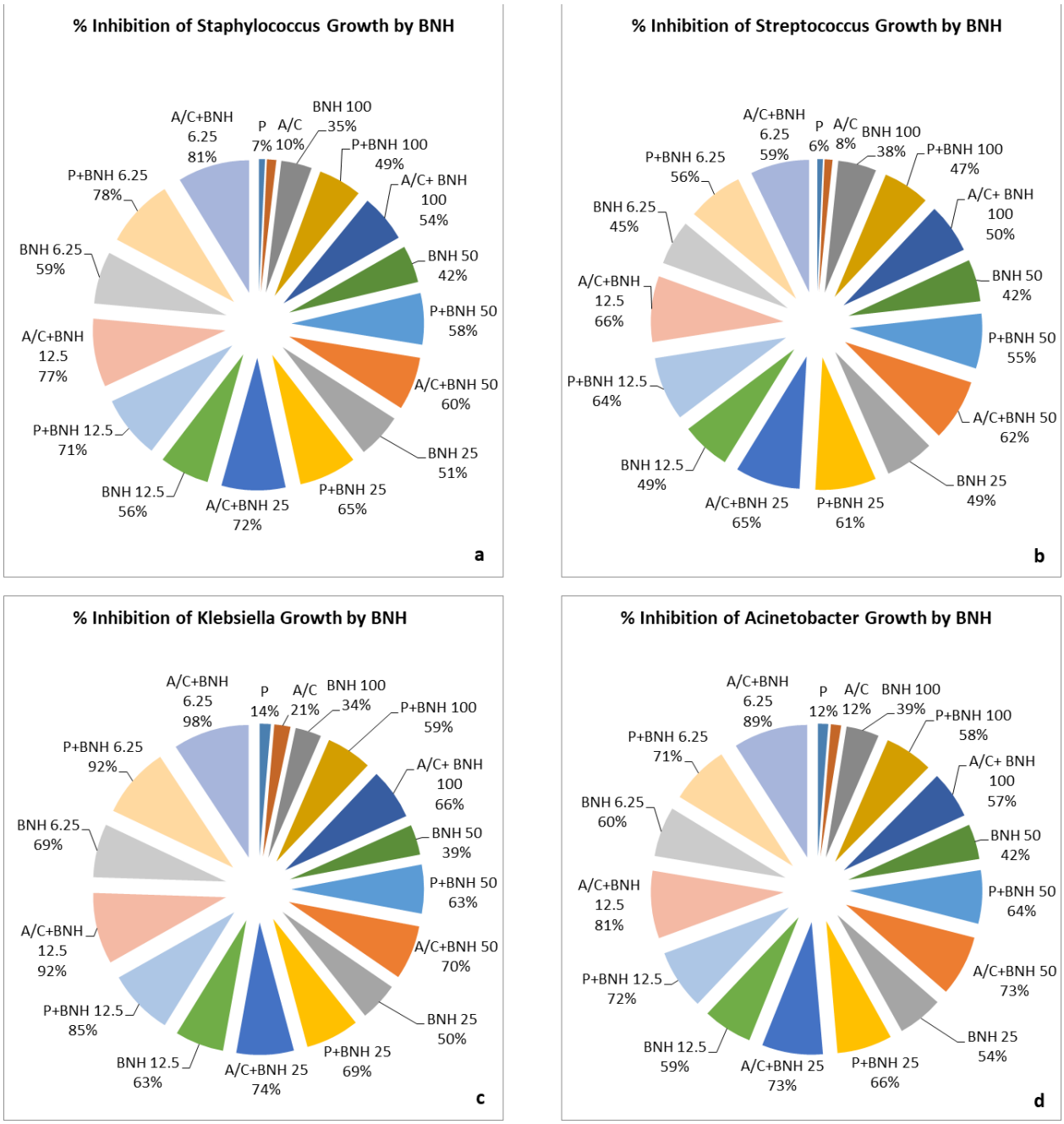

Figure 2 (a-d): Percentage inhibition of bacterial growth by mustard honey.

P=Penicillin, A/C=Amoxicillin-Clavulanic Acid, BNH= Mustard Honey, P+BNH=combination of penicillin and mustard honey, A/C+BNH=combination of amoxicillin-clavulanic acid and mustard 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.

Test Bacteria	Sample	MIC	MBC
Staphylococcus aureus	P + BNH	P 1µg/µl + BNH 12.5%	P 1µg/µl + BNH 6.25%
	A/C + BNH	A/C 1µg/µl + BNH 50%	A/C 1µg/µl + BNH 50%
Streptococcus pyogenes	P + BNH	P 1µg/µl + BNH 25%	N/A
	A/C + BNH	A/C 1µg/µl + BNH 12.5%	N/A
Klebsiella pneumoniea	P + BNH	P 1µg/µl + BNH 12.5%	P 1µg/µl + BNH 12.5%
	A/C + BNH	A/C 1µg/µl + BNH 25%	A/C 1µg/µl + BNH 12.5%
Acinetobacter baumannii	P + BNH	P 1µg/µl + BNH 12.5%	P 1µg/µl + BNH 12.5%
	A/C + BNH	A/C 1µg/µl + BNH 6.25%	A/C 1µg/µl + BNH 6.25%

Data represents the minimum concentrations at which the applied test samples showed inhibitory and bactericidal effect against the tested bacteria. N/A=No Effect, P+BNH=Combination of Penicillin and Mustard Honey, A/C+BNH=Combination of Amoxicillin-Clavulanic Acid and Mustard Honey. The other test conditions (P, A/C, BNH) did not have an effect.