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ORIGINAL CONTRIBUTION

Formulated honey benefits the action of penicillin and amoxiclav against resistant bacteria

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ABSTRACT

Background: Antibacterial properties of black seed honey, mustard honey and lychee honey have got scientifically acknowledged through laboratory investigation and thus, found in many literatures. Though their mixture is believed to have enhanced potential, no scientific attempt was made on evaluation of such mixture. The aim of the present study was therefore to not only screen the antibacterial efficacy but also to assess their potential to revive the weak antibiotics against resistant bacteria.

Methods: All three honey were uniformly mixed to generate a 1:1:1 honey formulation (FH) and paired it with penicillin (FH-P) and amoxiclav (FH-A/C) as separate combinations. Two gram-positive and two gram-negative resistant bacteria were collected from clinical isolates. Bacterial susceptibility was evaluated by agar well diffusion method and percentage of bacterial growth inhibition was observed through microdilution technique. Furthermore, the minimum inhibitory and bactericidal concentration was determined.

Results: The formulated honey showed an upward efficacy with dilution which demonstrated significant potential of honey in bacterial growth inhibition. FH-A/C displayed rigorous synergism against *K. pneumonia, S. aureus and S. pyogenes but failed against A. baumannii*. On the contrary, FH-P exhibited facilitated action of penicillin only against *S. pyogenes.*

Conclusion: The study confirmed that the efficacy of formulated mixture of three selected honey could be utilized for further investigation which may generate clues for natural adjuvant therapy with optimum formulation.

Keywords

Formulated Honey,
Black Seed Honey,
Lychee Honey,
Mustard Honey,
Antibacterial,
Well Diffusion,
Microdilution

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INTRODUCTION

Antibiotic resistance, a global health care issue, has raised a deep concern among the researchers, practitioners and the patients. While new bacteria are evolving at a pace, we are stumbling to tackle the existing bacteria due to their developing resistant pattern. The threat gets immense aided by the shortage of new antibiotics in the pipeline which to serve the immediate requirement. Uncountable reports have already confirmed the failure of penicillin and amoxicillin-clavulanic acid (amoxiclav) to treat the common infectious bacteria [1-3]. However, reports have also indicated that the newly evolved or mutated bacteria can be susceptible to these basic drugs [4,5]. If such potentials are confirmed, it will save both time and cost as well as open up scopes for new research. Therefore, it has become a time-demand to investigate a suitable way to revive these antibiotics. Utilizing natural agents as adjuvant for antibiotics could be an easiest one.

Antibacterial properties of raw honey have been well recognized in modern medical science. However, the degree of potential varies mostly upon the sources – the flowers. Raw honey is acknowledged after the name of the flower from which bees collect the nectar. Black seed honey, mustard honey and lychee honey are the most popular of them due to their pleasant taste as well as medicinal values [6]. Literatures have confirmed strong antibacterial efficacy of these three honeys against a wide range of bacteria [7-8]. Moreover, black seed honey and litchi honey were also reported to have synergistic effect on combined application with penicillin and amoxicillin-clavulanic acid [9,10]. Whereas, recent approaches in antibacterial research have been made to evaluate natural adjuvant therapy to boost the action of weak antibiotics against resistant bacteria [11], no attempt has been initiated to formulate honey mixture so as to act as an optimized natural adjuvant for those antibiotics. The present study thus, aimed to evaluate a mixture of black seed honey, mustard honey and lychee honey in facilitating the action of penicillin and amoxiclav against resistant bacteria.

METHODS

Collection and preparation of the sample

Black seed (*Nigella sativa*) honey, mustard (*Brassica Nigra*) honey and lychee (*Litchi chinensis*) honey were collected from the honey cultivators of Dinajpur (25.63°N, 88.65°E), Tangail (24°13′N 90°3′E) and Sunamganj districts (25.0715°N, 91.3992°E) of Bangladesh respectively. Each type of honey was taken at 250ml volume. The samples were sieved with a 0.5 mm mesh to separate the coarse material, pollen, bees wax and any other bee product or impurities. The filtered honey was then kept in an air-sealed glass container to protect the honey from accumulation of moisture on the surface. For the experiments, 50ml of each honey type were homogenously mixed with a ratio of 1:1:1, termed as formulated honey (denoted as FH).

Characterization of the samples

Physicochemical properties of the collected honeys were assessed qualitatively according to the standard procedures in previous studies [12-14].

Collection of bacterial isolates

Two gram-negative and two gram-positive resistant clinical isolates were obtained from the Center for Medical Biotechnology, Institute of Public Health, Bangladesh. The collected strains were *Klebsiella pneumonia* (ATCC 13883)*, Acinetobacter baumannii* (ATCC 17978)*, Staphylococcus aureus* (ATCC 6538) *and Streptococcus pyogenes* (ATCC 19615*) isolated from clinical samples.* The bacteria were overnight incubated in Muller Hinton Agar (MHA) and Nutrient Broth (NB) and preserved in 15% glycerol.

Bacterial Susceptibility Test

The well diffusion assay was employed to investigate bacterial susceptibility towards the test agents [15,16]. Two freshly prepared MHA plates (90mm) were use in this regard where bacterial suspension (10^7 CFU per ml) were uniformly streaked over using sterile cotton swab. Each plate was divided into four quadrants where an 6mm well was cut in each quadrant using sterile cork borer. The first plate was subjected to wells contained 20µl of the test samples as follows – sterile water (control), phenoxymethylpenicillin [Sanofi Aventis (BD) Ltd.], ampicillin-clavulanic acid [Sanofi Aventis (BD) Ltd.] and honey mixture. The second plate followed wells for control, blank, penicillin-formulated honey combination and amoxiclav-formulated honey combination. The plates were then set to incubation for 24h at 37°C ± 1°C and afterwards, the zones over each well were measured using a vernier calipers scale. The test was repeated three times to consider the variability.

Microdilution Assay

The method was carried out with six dilutions of the formulated honey in a 96-well microplate [17]. The stock mixture was considered as of 100% strength and two-fold serial dilution were performed to obtain 50%, 25%,

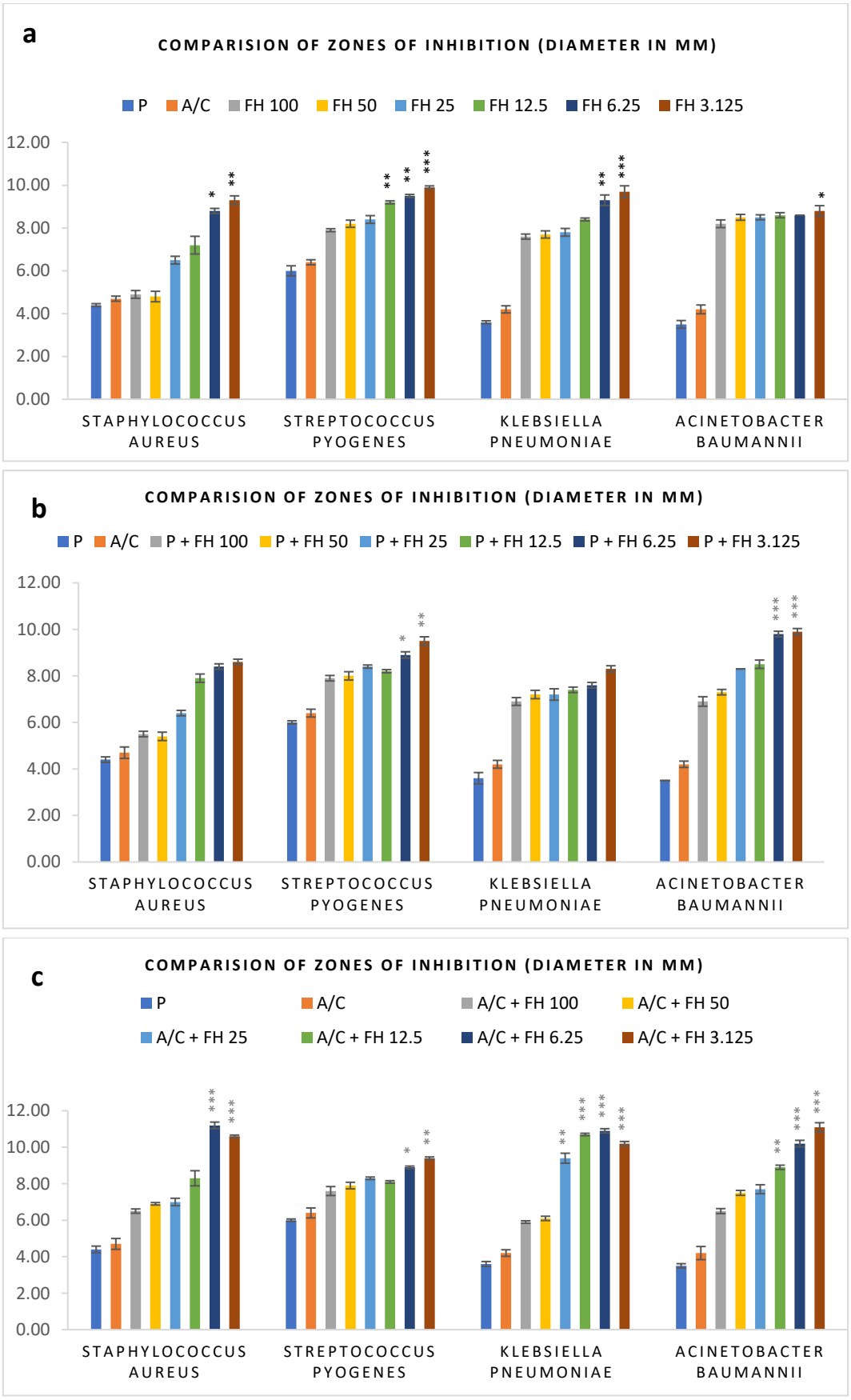

Figure 1 (a-c): Zones of inhibition of bacterial growth by (a) formulated honey and its combinations with standards (b) penicillin (c) amoxiclav, in agar well diffusion method. P = Penicillin; A/C = Amoxicillin + Clavulanic Acid; FH = Formulated mixture of black seed, mustard and litchi honey. Data represented as mean ± standard deviation, (n = 3); *p < 0.05, **p < 0.01, ***p < 0.001; Dunnett t-test (two sided).

12.5%, 6.25% and 3.125% strengths. These strengths were tested individually and in separate combination with penicillin and amoxiclav against the resistant isolates. Each well contained 10µl of bacterial suspension and 20µl of penicillin/amoxiclav/formulated honey in 300µl of NB, whereas, combination well contained 10µl of formulated honey and 10µl of penicillin/amoxiclav in place of individual test agent. The initial absorbance (T_0) of the test suspension was determined at 630nm using a microplate reader (Biobase EL-10A, China) and kept for incubation at 37°C ± 1°C. After 24h, another absorbance (T_{24}) was taken at the same wavelength. The difference of the two ($T_{24} - T_0$) was considered the absorbance for bacterial growth (Abs) from which the percentage of bacterial inhibition was calculated comparing with the same of control using following formula:

Percentage inhibition = $1 - (Abs._{test}/Abs._{control}) \times 100$

The method was performed in triplicate.

Minimum Inhibitory and Bactericidal Concentrations (MICs & MBCs)

From the microplate of microdilution assay, MIC was recorded by observing the wells with no visual turbidity at lowest concentration [10]. The observed well and its successive one (where applicable) were transferred to a fresh MHA plate and incubated for another 24h at 37°C ± 1°C. If the sample produced no visual colony was considered as MBC [18].

Statistical Analysis

Data were expressed as mean (n=3) ± standard deviation. To compare all groups with the control Dunnett t test was performed. The statistical analysis was carried out with one-way ANOVA test using SPSS v.24 where a p value less than 0.05 was considered significant.

RESULTS

From agar well diffusion assay, it was observed that honey tended to show better efficacy in lower strengths following a linear dilution-dependent increase pattern (Figure 1a-1c). The same was observed in microdilution assay too (Figure 2a-2b, 3a-3b).

In agar well diffusion assay, the formulated honey in its individual use exhibited largest zone of inhibition against *S. pyogenes* (9.9mm at 3.125%) followed by *K. pneumoniae* (9.7mm at 3.125%). When applied together with penicillin, the combination worked best against *A. baumannii* (9.9mm, 3.125%) in comparison to its individual application (8.8mm, 3.125%). However, the conjugation found to reduce the inhibition of other three bacteria in comparison to that of formulated honey itself. On the contrary, amoxiclav-formulated honey coupling displayed synergistic antibacterial potential maximum on *S. aureus* (11.2mm, 6.25%), *K. pneumoniae* (10.9mm, 6.25%), A. *baumannii* (11.1mm, 3.125%) but except for *S. pyogenes* (9.4mm, 3.125%) where it was found to reduce the activity. Against all bacteria, penicillin and amoxiclav showed negligible inhibition.

In microdilution assay, percentage of inhibition for individual bacterial growth was calculated. In case of *S. aureus* (Figure 2a), the formulated honey demonstrated a higher potential in inhibition with amoxiclav (55%, 6.25%) than individual use (37%, 3.125%) though, with penicillin contrarily decreased the potential (33%, 3.125%). But against *S. pyogenes*, despite the fact that FH produced higher inhibition (39%, 3.125%) comparing to that of S. aureus, could not exhibit such potential with amoxiclav (35%, 6.25%). *K. pneumoniae* growth was found to firmly inhibited by FH at 3.125% (42%) and FH-Amoxiclav at the same strength (51%). When paired with penicillin, both *S. pyogenes* and *K. pneumoniae* showed less susceptibility towards it than the FH itself. However, penicillin's action was facilitated by FH only against *A. baumannii* at 3.125% (42%) which was higher than FH's maximum efficacy (35%, 12.5%) as individual agent. Alongside, the FH-Amoxiclav combination also peaked at 12.5% (56%). Penicillin and amoxiclav could demonstrate mild efficacy against gram-positive bacteria only.

Table 1 shows the MIC and MBC values against the different bacterial strains. With A/C+FH combinations the MIC was achieved at 3.125% for all except *A. baumannii* (6.25%). MBC for *A. baumannii* was recorded at 12.5% whereas MBCs for both *K. pneumoniea* and *S. aureus* were found at 6.25% FH. No MIC or MBC were obtained for *S. pyogenes* either with the standards.

DISCUSSION

Findings suggests that the formulated honey possesses a dilution-gradient efficacy relationship with lower strength demonstrating higher efficacy, which supported previous studies [19]. Moreover, it can be confirmed that the degree of antibacterial potential exhibited by this mixture of honey was noteworthy. Regarding the combinations, FH-Penicillin showed synergistic efficacy against *A. baumannii* but not against other bacteria. On the contrary, FH-Amoxiclav displayed similar action against all bacteria except *S. pyogenes*.

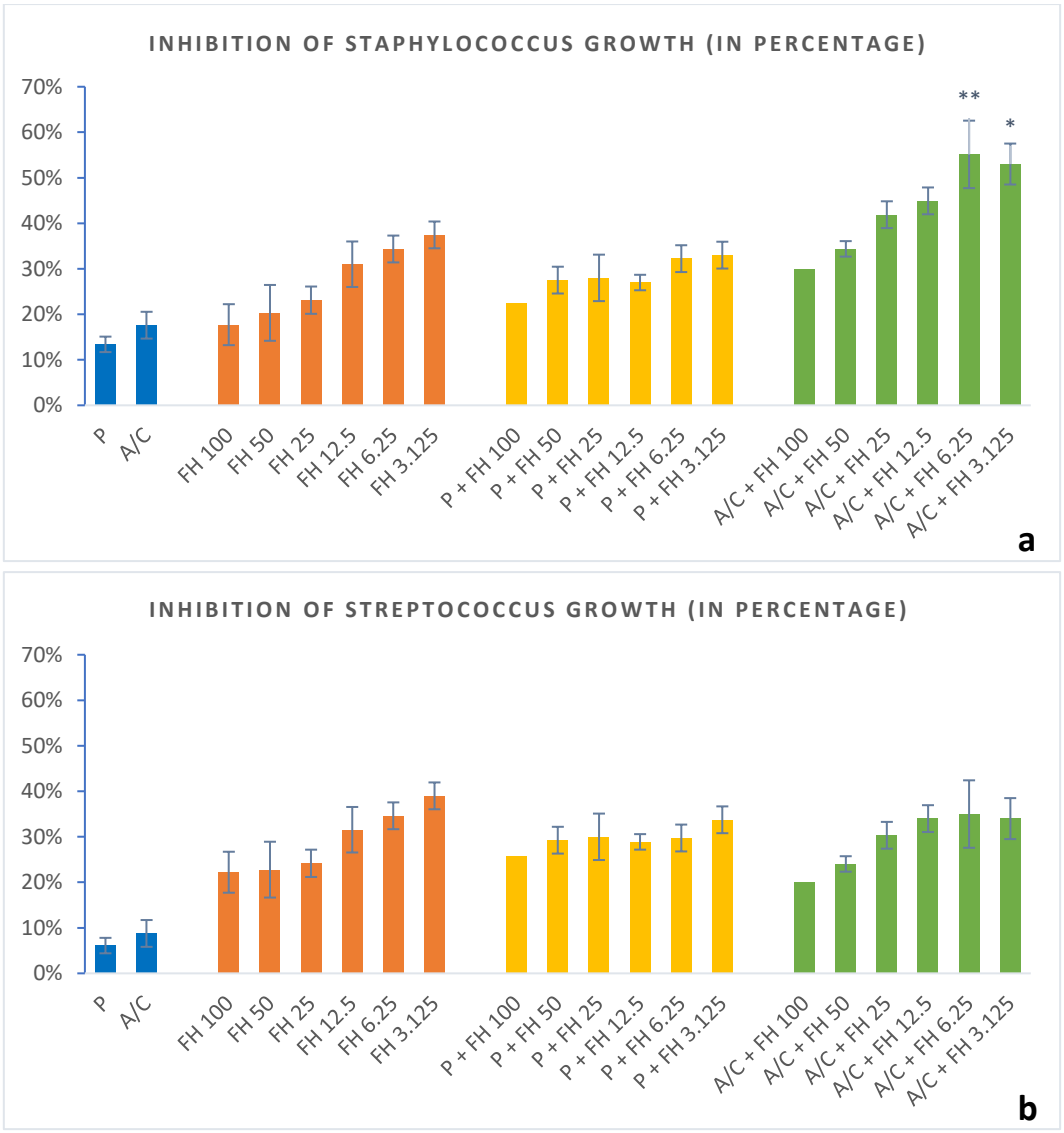

Figure 2 (a-b): Percentage of inhibition of bacterial growth (gram-positive) by formulated honey and its combinations with standards in microdilution method. (a) *Staphylococcus aureus* (b) *Streptococcus pyogenes*.
P = Penicillin; A/C = Amoxicillin + Clavulanic Acid; FH = Formulated mixture of black seed, mustard and litchi honey. Data represented as percentage of inhibition as mean ± standard deviation, (n = 3); *p < 0.05, **p < 0.01; Dunnett t-test (two sided).

Honey is a complex natural antibacterial agent consisting of contributory factors like osmolarity [20,21], hydrogen peroxide [22], polyphenols [23], antioxidants [24] antibiotic peptides [25], and Maillard reaction products [24]. In diluted honey, glucose oxidase (GOX) enzyme generates hydrogen peroxide, which interacts with bacterial cell proliferative signals and eventually prevent bacterial growth [26-28]. Moreover, the antibacterial properties have been attributed to Def-1, an antibacterial peptide, possessing broad spectrum antibiotic effect [26]. The antibacterial activity of honey is also credited to the presence of other constituents like methylglyoxal (MGO) and its precursor dihydroxyacetone (DHA) [29].

Thymoquinone and melanin are the main active components of black seed honey that contribute to its antimicrobial property [30]. Honey derived from mustard also possesses certain pharmacological properties that are attributed to its nutritional elements- polyphenol, vitamin C, flavonoids and certain heavy metals [31]. Similar compounds apart from antioxidants have been also reported for litchi honey which may have contributed to its antibacterial properties [32].

Beta-lactam antibiotics exerts their action by blocking transpeptidase enzyme, a precursor for cross linkage of peptidoglycan in both gram-positive and gram-negative

Figure 3 (a-b): Percentage of inhibition of bacterial growth (gram-negative) by formulated honey and its combinations with standards in microdilution method (a) *Klebsiella pneumoniae* (b) *Acinetobacter baumannii*.
P = Penicillin; A/C = Amoxicillin + Clavulanic Acid; FH = Formulated mixture of black seed, mustard and litchi honey. Data represented as percentage of inhibition as mean ± standard deviation, (n = 3); *p < 0.05, **p < 0.01; Dunnett t-test (two sided).

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

Test Bacteria	***Sample***	***MIC***	***MBC***
Staphylococcus aureus	P + FH	N/A	N/A
	A/C + FH	A/C 1µg/µl + FH 3.125%	A/C 1µg/µl + FH 6.25%
Streptococcus pyogenes	P + FH	N/A	N/A
	A/C + FH	N/A	N/A
Klebsiella pneumoniea	P + FH	N/A	N/A
	A/C + FH	A/C 1µg/µl + FH 3.125%	A/C 1µg/µl + FH 6.25%
Acinetobacter baumannii	P + FH	P 1µg/µl + FH 3.125%	N/A
	A/C + FH	A/C 1µg/µl + FH 6.25%	A/C 1µg/µl + FH 12.5%

Data represent the minimum concentrations, at which the applied test samples showed inhibitory and bactericidal effect against the tested bacteria. N/A = No effect, P+FH = Combination of penicillin and mixed honey, A/C+FH = Combination of amoxicillin–clavulanic acid and mixed honey. The other test conditions (P, A/C, and FH alone) did not have an effect.

bacteria [33]. Consequently, cell becomes ruptured due to notwithstanding the osmotic pressure [34]. Resistance to penicillin (once most effective β-lactam antibiotic) occurs due to the target modification of peptidoglycan-binding-proteins in gram-positive bacteria [35], while gram-negative bacteria possessing a double membrane resists the entry of penicillin across it [36]. Moreover, it hydrolyzes the β-lactam ring [36]. A third-generation penicillin, amoxicillin, used to show higher stability to β-lactamase and perform with better efficacy [34]. However, it also is not out of the scope of cleavage of beta-lactam ring by the bacteria. Combination of amoxicillin and clavulanic acid (beta-lactamase inhibitor). As like the inherent characteristics of clavulanic acid of not having any direct bactericidal properties [37], some compounds of honey could have contributed in similar mechanism to obtain the reverse effect of penicillin and amoxiclav.

CONCLUSION

The formulated mixture of these three honeys remarkably inhibited both the gram-positive and gram-negative bacteria. its combination with penicillin and amoxiclav also proved to have synergistic potential, however, at this stage of the study the underlying mechanism is unknown. Further investigation shall be undertaken to identify the responsible bioactive compounds.

Abbreviations

PEN: Penicillin; A/C = Amoxicillin + Clavulanic Acid; FH = Formulated mixture of black seed, mustard and litchi honey; MHA: Muller Hinton Agar; NB: Nutrient Broth; ATCC: American Type Culture Collection.

Ethics Approval and Consent to Participate

The work was exempt for ethical approval. All experiments were performed in accordance with the Institutional Standard Microbial Testing Procedures (Ref. no. IPSDRLAB/ ISMTP/01/19) of Institute for Pharmaceutical Skill Development and Research, BD.

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Consent for Publication

Not applicable.

Authors' Contributions

This work was carried out in collaboration between both authors, contributed equally.

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Availability of Data and Materials

Available from corresponding author on valid request.

Competing Interests

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

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