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Original Research Article

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

Rozely Hossain[1], M. S. Rahman[2], M. A. Rayhan[1], Kashfia Nawrin[3], Mohammad M. Billah[3]*, M. R. Habib[4]

[1]Department of Genetic Engineering and Biotechnology, East West University, Bangladesh
[2]Department of Microbiology, Jahangirnagar University, Bangladesh
[3]Department of Pharmacology, Institute for Pharmaceutical Skill Development and Research, Bangladesh
[4]Directorate General of Drug Administration, Bangladesh

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***Correspondence:**
Mr. Mohammad M. Billah,
E-mail: mustakimbillah@hotmail.com

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 Ranunculacea 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.[2] In general, honey has 1% to 5% water and 95% to 99% sugar containing fructose, glucose, maltose, sucrose and isomaltose.[3] 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.[4] 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.[3,5] 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.[1] Apart from that, honey has found to have antimicrobial action against a broad spectrum of pathogenic bacteria and fungi in many laboratory studies.[3] 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.[3] Some studies suggested that the osmotic effect, acidity, hydrogen peroxide and phytochemical factors optimize the antimicrobial activity in honey.[3,6]

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.[7] 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 spectro-photometer was used for adjusting the absorbance at 580 nm and diluted to have viable cell count of 10^7 CFU/ml.[8]

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 phenoxymethylpenicillin (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.[9]

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined through the method described by Patton et al. with slight modifications.[10,11] 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, phenoxymethylpenicillin, 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 recordte the initial value (T0). 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:

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

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.[12] 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 phenoxymethylpenicillin 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.

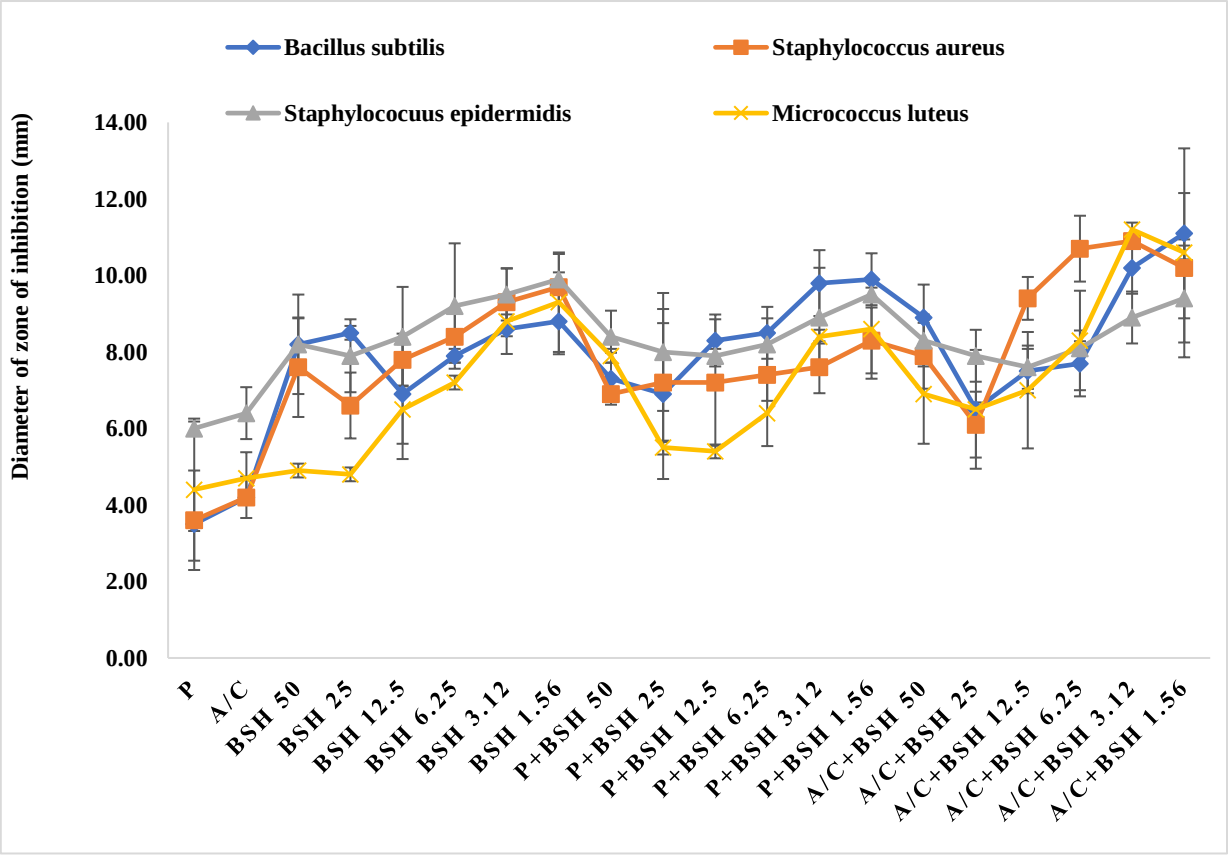

Figure 1: Relationship between doses and corresponding zones of inhibition by well diffusion method.

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.

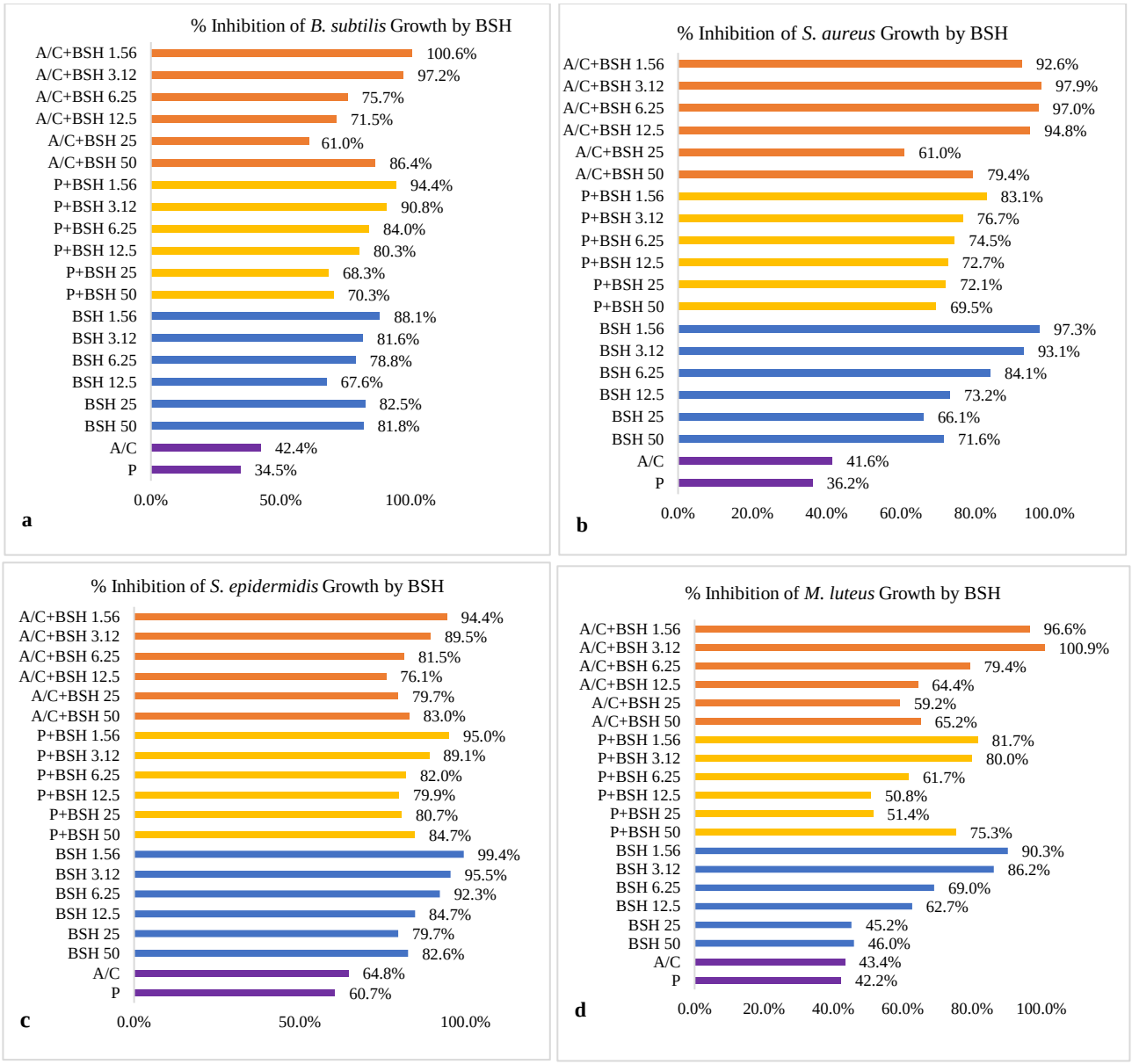

Figure 2 (a-d): Percentage inhibition of bacterial growth by black seed honey.

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.

Test bacteria	Sample	MIC	MBC
Bacillus subtilis	P	N/A	N/A
	A/C	N/A	N/A
	BSH	BSH 1.56%	N/A
	P + BSH	P 1 µg/µl + BSH 1.56%	P 1 µg/µl + BSH 1.56%
	A/C + BSH	A/C 1 µg/µl + BSH 3.12%	A/C 1 µg/µl + BSH 1.56%
Staphylococcus aureus	P	N/A	N/A
	A/C	N/A	N/A
	BSH	BSH 1.56%	BSH 1.56%
	P + BSH	P 1 µg/µl + BSH 1.56%	N/A
	A/C + BSH	A/C 1 µg/µl + BSH 3.12%	A/C 1 µg/µl + BSH 3.12%

Continued.

Test bacteria	Sample	MIC	MBC
Staphylococcus epidermidis	P	N/A	N/A
	A/C	N/A	N/A
	BSH	BSH 1.56%	BSH 1.56%
	P + BSH	P 1 µg/µl + BSH 1.56%	N/A
	A/C + BSH	A/C 1 µg/µl + BSH 1.56%	N/A
Micrococcus luteus	P	N/A	N/A
	A/C	N/A	N/A
	BSH	BSH 1.56%	N/A
	P + BSH	P 1 µg/µl + BSH 1.56%	N/A
	A/C + BSH	A/C 1 µg/µl + BSH 1.56%	A/C 1 µg/µl + BSH 3.12%

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=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.

DISCUSSION

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.[13] Phenoxy-methyl penicillin, exhibits bactericidal effect against penicillin-sensitive microorganisms during the stage of active multiplication.[14] But the beta-lactam ring of phenoxymethylpenicillin 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.[15] On the other hand, amoxicillin is a semisynthetic penicillin, works by the same mechanism of action.[16] The combination of amoxicillin and clavulanic acid exerts bactericidal effect against a wide range of gram-positive and gram-negative bacteria.[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.[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.[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.[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 phenoxymethylpenicillin 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.

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REFERENCES

1. Forouzanfar F, Bazzaz BS, Hosseinzadeh H. Black cumin (*Nigella sativa*) and its constituent

(thymoquinone): a review on antimicrobial effects. Iran J Basic Med Sci. 2014;17(12):929-38.
2. Hegazi AG, Al Guthami FM, Al Gethami AFM, Allah FMA, Saleh AA, Fouad EA. Potential antibacterial activity of some Saudi Arabia honey. Vet World. 2017;10(2):233-237.
3. Olaitan PB, Adeleke OE, Ola IO. Honey: a reservoir for microorganisms and an inhibitory agent for microbes. Afr Health Sci. 2007;7(3):159-65.
4. Aurongzeb, M, Azim, MK. Antimicrobial properties of natural honey: a review of literature. Pak J Biochem Mol Biol. 2011;44(3):118-24.
5. 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 Cir Bras. 2018;33(6):518-23.
6. Rayhan A, Yousuf SA, Rayhan J, Khengari EM, Nawrin K, Billah MM. Black seed honey—A powerful ingredient of prophetic medicine; its neuropharmacological potential. J Apither. 2019;5(2):18-26.
7. Sharma R1, Sharma CL, Kapoor B. Antibacterial resistance: Current problems and possible solutions. Indian J Med Sci. 2005;59(3):120-9.
8. Mostafa AA, Al-Askar AA, Almaary KS, Dawoud TM, Sholkamy EN, Bakri MM. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi J Biol Sci. 2018;25(2):361-6.
9. National Committee for Clinical Laboratory Standards (2017). Methods for determining bactericidal activity of antimicrobial agents. Tentative Guidelines, M26-TNCCLS, Villanova, PA. 2017.
10. Patton T, Barett J, Brennan J, Moran N. Use of a spectrophotometric bioassay for determination of microbial sensitivity to manuka honey. J Microbiol Methods. 2005;64:84-95.
11. Gambogou B, Khadimallah H, Bouacha M, Ameyapoh YA. Antibacterial activity of various honey monofloral and polyfloral from different region of Algeria against uropathogenic Gram Negative Bacilli. J Apither. 2018;4(1):1-8.
12. Bouacha M, Ayed H, Grara N. Honey bee as alternative medicine to treat eleven multidrug-resistant bacteria causing urinary tract infection during pregnancy. Sci Pharm. 2018;86(2):14.
13. Yocum RR, Rasmussen JR, Strominger JL. The mechanism of action of penicillin. Penicillin acylates the active site of Bacillus stearothermophilus D-alanine carboxypeptidase. J Biol Chem. 1980;255(9):3977-86.
14. National Center for Biotechnology Information. PubChem Database. Penicillin v, CID=6869. Available at https://pubchem.ncbi.nlm.nih.gov/compound/Penicillin-v. Accessed on 28 October 2019.
15. Venugopalan V, Klinker, KP. Chapter 29: Cell Wall Inhibitors. In Lippincott® Illustrated Reviews: Pharmacology. 7th ed. Wolters Kluwer; 2019: 1047-1058.
16. Akhavan BJ, Vijhani P. Amoxicillin. In: Stat Pearls. Treasure Island (FL): Stat Pearls Publishing. Available at: https://www.ncbi.nlm.nih.gov/books/NBK482250/. Accessed on 28 October 2019.
17. Finlay J, Miller L, Poupard JA. A review of the antimicrobial activity of clavulanate. J Antimicrob Chemother. 2003;52(1):18-23.
18. Stapleton P, Wu PJ, King A, Shannon K, French G, Phillips. Incidence and Mechanisms of Resistance to the Combination of Amoxicillin and Clavulanic Acid in Escherichia coli. Antimicrob Agents Chemother. 1995;39(11):2478-83.
19. Hamdy AA, Ismail HM, Al-Ahwal AM, Gomaa NF. Determination of flavonoid and phenolic acid contents of clover, cotton and citrus floral honeys. J. Egypt. Public Health Assoc. 2009;84:245-59.
20. Burns K. Health benefits of prophetic condiments: part one of five. Islam Online Arch 2000. Available at: https://archive.islamonline.net/?p=15586. Accessed on 24 May 2019.
21. Alvarez-Suarez JM, Giampieri F, Battino M. Honey as a source of dietary antioxidants: structures, bioavailability and evidence of protective effects against human chronic diseases. Curr Med Chem. 2013;20:621-38.
22. Cushnie TP, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents. 2005;26:343-56.

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