# Evaluation of Antibacterial Activities of Aqueous Extract of Black Pepper *(Piper nigrum L)* Seeds against the Gram Positive *Staphylococcus aureus and* Gram-Negative *Escherichia coli*

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Antibiotic resistance (AMR) represents a serious threat to public health and poses challenges in disease prevention and treatment despite various efforts to combat it. Evaluation of the in vitro antibacterial activity of aqueous extracts of black pepper seeds (Piper nigrum L.) against two infectious pathogens: Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. The Department of Pharmacology and Therapeutics and the Department of Microbiology of Mymensingh Medical College conducted the study from July 2022 to June 2023. The antibacterial activity of Aqueous black pepper seed extract (ABPE) was evaluated at different doses using disk diffusion and broth dilution methods. The extract was prepared using 10.0% dimethyl sulfoxide (DMSO) and water as solvent. The commonly used antibiotic ciprofloxacin was used in the broth dilution method and the results were compared with those for aqueous extracts. To confirm a more precise range of antimicrobial susceptibility of the extracts, ABPE was used at seven different concentrations (100, 80, 60, 40, 20, 10 and 5 mg/mL). Selected concentrations were then used as needed. ABPE showed an inhibitory effect on the above bacteria at doses of 90 mg/ml and higher. The Minimum inhibitory concentration (MIC) values for *Escherichia coli* and *Staphylococcus aureus* were 85 and 90 mg/ml ABPE, respectively. The MIC of ciprofloxacin against *Staphylococcus aureus* and *Escherichia coli* was currently 1µg/ml. The MIC of ciprofloxacin was lowest for the organisms tested compared to the MIC of ABPE. This work clearly demonstrates the antibacterial sensitivity of *Staphylococcus aureus* and *Escherichia coli* to an aqueous extract of black pepper seeds.

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**Key words:** Antibacterial effects, Black pepper seeds, *Staphylococcus aureus*, *Escherichia coli*, Disc diffusion, Aqueous extract

## Introduction

Today, infectious illness ranks third in industrialized countries and is the second biggest cause of death globally. Seventeen (17) million individuals every year die from bacterial infections worldwide. Numerous people contact with antibiotic-resistant germs each year, and as a result, many of them end up dying from their infections[1]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for many deaths. The fifth most common killer in the world is enteric diseases, which are 70.0% food-borne. Diarrheal illness affects predominantly children under the age of five in its 1.5 billion annual cases. *E. coli*, *Campylobacter* and *Brucella* are among the 31 organisms that are known to cause 99.0% of these illnesses[2]. Antibiotic resistance is a naturally occurring process. The foundation of contemporary medicine, antibiotics have made significant contributions over the past 50 years to the advancement of healthcare. Since resistance is an unavoidable result of the medication selection pressure, it can be gradually slowed down but not totally eliminated[3].

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According to the WHO, more than 80.0% of the world's population now relies mostly on traditional medicines made from plants for their medical needs[4]. Since the late 19th century, scientific studies have shown that various spices, herbs, and their components have antibacterial characteristics. Due to the rising danger of contacting an illness with an antibiotic-resistant microbe as well as the side effects and after effects of antibiotics, there has recently been an increased interest in the identification of novel natural antimicrobials[5]. Since ancient times, black pepper (Piper nigrum L., family Piperaceae), known as the "king of spices", has been a popular spice. It is a smooth, woody blooming shrub that has spread rapidly over Bengal, Malaysia, Indonesia, Brazil, Assam, Vietnam, China, Thailand, Sri Lanka and India[6]. In Bangladesh, it is known as "Golmarich"[7]. The results of the phytochemical studies on P. nigrum showed that it contains a range of phytochemicals. The first substance from the Piperaceae family to be identified that was pharmacologically active was piperine. Researchers have identified various chemicals such as phenolics, flavonoids, alkaloids, amides, steroids, lignans, neolignans, terpenes, and chalcones[8]. Black pepper, with its principal ingredient piperine, has various pharmacological effects, including anti-inflammatory, anti-diarrheal, antioxidant and antibacterial properties, due to its ability to modify membrane permeability[9]. Previous studies have demonstrated the antibacterial properties of pepper and secondary metabolites of Piper nigrum. The purpose of this study is to determine whether black pepper seed extracts exhibit antibacterial activity in vitro against several common food borne pathogens such as *Staphylococcus aureus* and *Escherichia coli.*

The aim of the present study is to evaluate the antimicrobial properties of aqueous black pepper seed extract against various food-borne diseases. This study could highlight the potential of black pepper seeds as medicinal plants in the fight against drug-resistant bacteria that pose a serious threat to human health. It could also be useful for evaluating the response of different bacterial species to synthetic antibiotics.

## Methods

This experimental study was conducted from July 2022 to June 2023 in the Department of Pharmacology and Therapeutics in collaboration with the Department of Microbiology, Mymensingh Medical College, Bangladesh.

**Preparation of Aqueous black pepper seeds Extract (ABPE ):**

Black pepper (*Piper nigrum L.*) seeds were cleaned and washed.

⇩

Shaded-dried at room temperature for 2 weeks.

⇩

Pulverized by a grinder. Grounded Black pepper seeds (50gm) dissolved in 500 ml of Distilled water

⇩

Kept it for 3 days at room temperature and away from the light.

⇩

The Methanol extract was then filtered by using cotton cloth & then again filtered by passing through Whatman No.1 filter paper.

⇩

The resulting filtrate was collected in previously tared sterilized conical flask and Reduced to dryness by removing the solvent in an air-dried oven at 40°C.

⇩

Dried extracts were then exposed to ultraviolet light (UV) for 24h to sterilize.

⇩

Then 10% DMSO was added in each bottle to get the final concentration of 200 mg/ml.

⇩

This extract was stored in refrigerator at $4^0$C until further use.

After obtaining the approval of protocol from the Institutional Review Board (IRB) of MMC (Memo no: MMC/IRB/2023/547 Dated: 04/01/ 2023) this study was performed.

*Tested bacterial organisms*: Two bacterial strains were used in this study, *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). These two test organisms were reference strains and their pure cultures were collected by the Department of Microbiology, Mymensingh Medical College, Bangladesh.

Upkeep of bacterial culture and creation of inoculums: Pure cultures were routinely maintained and supplemented on inclined nutrient agar plates. Cultures were plated on sterile nutrient agar plates, incubated at 37°C for 24 hours, and then cooled to 4°C. To avoid any contamination, the bacterial cultures were renewed every one to two weeks. To prepare inoculums, a pure bacterial culture was grown in nutrient broth overnight at 37°C.

Collection of plant material: Black Pepper seeds were bought from local market of Mymensingh Sadar, Mymensingh, Bangladesh.

Powdered black pepper seed preparation: According to the method outlined by Ali M et al.[10], black pepper seeds powder was made. Black pepper seeds that were mature and fresh were purchased in the Mymensingh Sadar local market in Mymensingh, Bangladesh. Under flowing tap water, seeds were carefully cleaned two or three times. The seeds were then dried for 14 days at room temperature in the open, away from direct sunshine, before being repeatedly pulverized into a fine powder with the use of a domestic electric grinder and sieving.

*Preparation of Aqueous stock solution*

For preparation of aqueous stock solution, 1 gm of aqueous extract powder was dissolved in 5ml 10.0% dimethyl sulfoxide (DMSO) to get a concentration of 200 mg/ml. 200 mg/ml concentration of extract was considered as stock solution. For preparation of working solution from the above stock solution different concentration such as 100 mg/ml, 80 mg/ml, 60 mg/ml, 40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml were prepared by mixing with appropriate volumes of 10.0% DMSO. (Note: 200 mg/ml concentration of extract was considered as stock solution and kept for further use.)

*Disc diffusion method for determining the sensitivity of microorganisms to antibiotics* - Utilizing the Kirby-Bauer Disc diffusion method, the test was run. Whatman No. 1 filter paper was used to create and sterilize a 6 mm-diameter filter paper disc. A sterile cotton swab was dipped into bacterial suspension by matching to 0.5 McFarland turbidity strand for each isolate and streaked it in three directions on the surface of Mueller Hinton Agar plates then left for 5-10 minutes on room temperature. The blank discs were aseptically put over the Mueller Hinton agar plates containing the test microorganisms using ethanol-dipped and flamed forceps. Then with the help of micropipette 10 µ L of 100 mg/ml, 80 mg/ml, 60 mg/ml, 40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml concentrations of Aqueous extracts were transferred to different disc aseptically. Plates were incubated at 37ºC for 24 hours. As a negative control, 10 µL of 10.0% DMSO were poured to a sterile filter paper disc. The diameter of the zone of inhibition was measured in millimeters using a ruler after 24 hours, and the findings were recorded (Result shown in Figure 1 and 2). According to Alves et al.[11] the antibacterial activity findings were represented in terms of the width of the zone of inhibition, with a zone smaller than 9 mm being regarded inactive, 9-12 mm being partly active, 13-18 mm being active and >18 mm being extremely active.

Table I: Preparation of the ABPE solutions of different concentration

| Sl. No. | Amount of solution (ml) taken from Aqueous stock solution | Amount of 10.0% DMSO (ml) | Concentration (mg/ml) |
|---|---|---|---|
| 1 | 1.0 | 1 .0 | 100 |
| 2 | 0.8 | 1.2 | 80 |
| 3 | 0.6 | 1.4 | 60 |
| 4 | 0.4 | 1.6 | 40 |
| 5 | 0.2 | 1.8 | 20 |
| 6 | 0.1 | 1.9 | 10 |
| 7 | 0.05 | 1.95 | 05 |

Using the broth dilution technique, the minimum inhibitory concentration (MIC) of aqueous black pepper extract against *Staphylococcus aureus* and *Escherichia coli* was determined: Preparation of different concentrations of working Aqueous black pepper seed extract (ABPE) solution- Set-I: working solution was made by mixing 1 ml of ABPE stock solution with 1ml of Nutrient broth (NB). So, the total amount became 2 ml. As stated previously, 1 ml of ABPE stock solution contained 200 mg extract. Now 2 ml (1 ml ABPE solution + 1 ml NB) of the preparation contain 200 mg of extract. So, 1 ml of the preparation contained (200÷2) mg = 100 mg of extract. The final concentration was 100 mg/ml. Similarly, Set-II working ABPE solution was made by mixing 0.9 ml of ABPE stock solution with 1.1 ml of nutrient broth medium. So, the total amount became 2 ml. As stated previously, 1 ml of ABPE stock solution contained 200 mg extract. So, 0.9 ml of ABPE stock solution contained (200 × 0.9) mg = 180 mg of extract. Now 2 ml (0.9 ml ABPE solution + 1.1 ml NB) of the preparation contain 180 mg of ABPE. So, 1 ml of the preparation contains (180 ÷ 2) = 90 mg ABPE. Now the concentration of this dilution was 90mg/ml. Then Sets- III, IV, V, VI, VII, VIII, IX, X and XI respectively of working ABPE solutions were made in different test tubes by mixing measured amount of ABPE stock solution with measured amount of nutrient broth medium. The concentrations of these sets were 80 mg/ml, 70 mg/ml, 60 mg/ml, 50 mg/ml, 40 mg/ml, 30 mg/ml, 20 mg/ml, 10 mg/ml and 5 mg/ml respectively (Table II),

Set-XII (Control-1) was made with ABPE stock solution. Set-XIII (Control-2) was made with nutrient broth medium (inoculated with bacterial suspension). Set-XIV (Control-3) was made with nutrient broth medium (not inoculated with bacterial suspension) in test tubes.

*Inoculation of bacterial suspension to different concentrations of working ABPE Solutions with nutrient broth media in test tubes-*

After matching the turbidity of bacterial suspension with 0.5 McFarland standards, 20 µl or one drop (0.02 ml) of bacterial suspension of *Staphylococcus aureus* and *Escherichia coli* were separately added to each 10ml preparations of different concentrations of working ABPE solutions in separate test tubes. These inoculums were also added to the controls-1 and 2 except Control-3. The test tubes were marked set wise with black marker pen and were placed in the incubator at 37ºC for 18-24 hours.

Testing antimicrobial activity of a standard antibiotic: The test microorganisms i.e., *S. aureus* and *E. coli* were also tested for their activity against the antibiotic Ciprofloxacin (Inj. 200mg/ 100ml) by broth dilution method.

Table II: Composition and different concentrations of working ABPE solutions and the controls

| Number of sets | ABPE stock solution (ml) | Nutrient broth medium (ml) | Total (ml) | Concentration of ABPE (mg/ml) | Test organism (µl) |
|---|---|---|---|---|---|
| Set- I | 1.0 | 1.0 | 2.0 | 100 | 20 |
| Set- II | 0.9 | 1.1 | 2.0 | 90 | 20 |
| Set- III | 0.8 | 1.2 | 2.0 | 80 | 20 |
| Set- IV | 0.7 | 1.3 | 2.0 | 70 | 20 |
| Set- V | 0.6 | 1.4 | 2.0 | 60 | 20 |
| Set- VI | 0.5 | 1.5 | 2.0 | 50 | 20 |
| Set- VII | 0.4 | 1.6 | 2.0 | 40 | 20 |
| Set-VIII | 0.3 | 1.7 | 2.0 | 30 | 20 |
| Set-IX | 0.2 | 1.8 | 2.0 | 20 | 20 |
| Set-X | 0.1 | 1.9 | 2.0 | 10 | 20 |
| Set-XI | 0.05 | 1.95 | 2.0 | 5 | 20 |
| Set-XII C-1 | 02.0 | - | 2.0 | 1000 | 20 |
| Set-XIII C-2 | - | 2.0 | 2.0 | - | 20 |
| Set-XIV C-3 | - | 2.0 | 2.0 | - | - |

**Results**

According to zone of inhibition parameter, it is clearly observed that there is definite antibacterial activity of ABPE against both *Staphylococcus aureus* and *Escherichia coli*.

Aqueous extract of Black Pepper seeds Extract showed varying degrees of antibacterial activity starting from 100 mg/ml. Between two test organisms, *Staphylococcus aureus* was found to be most susceptible to ABPE (18mm) at 100 mg/ml concentration. Negative control (disc containing only 10.0% DMSO) showed no zone against any bacteria. The MICs of ABPE against tests organisms were determined through broth dilution method and the MICs were 90 mg/ml for *Staphylococcus aureus*, 85 mg/ml for *Escherichia coli*. The outcomes of the earlier tests were likewise supported by the subculture research. The results of Aqueous seeds extract of Black pepper extracts were also compared against a standard antibiotic Ciprofloxacin by broth dilution technique (shown in Figure 4). From these experiments it can be stated that, the MICs of Ciprofloxacin against *Staphylococcus aureus* and *Escherichia coli* were same (1µg/ml).

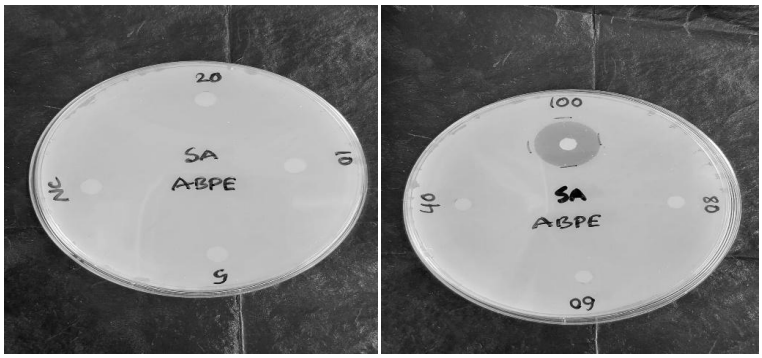


Figure 1: Antibacterial sensitivity testing of ABPE against *Staphylococcus aureus*

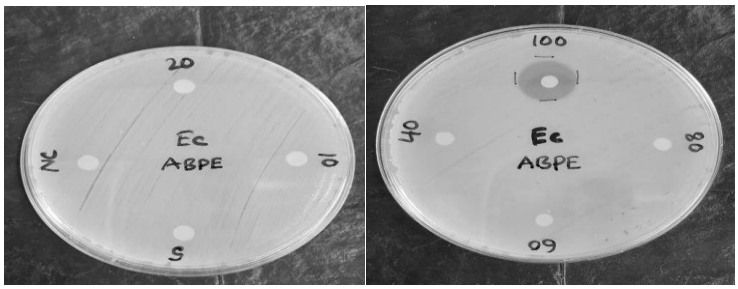


Figure 2: Antibacterial sensitivity testing of ABPE against *Escherichia coli*

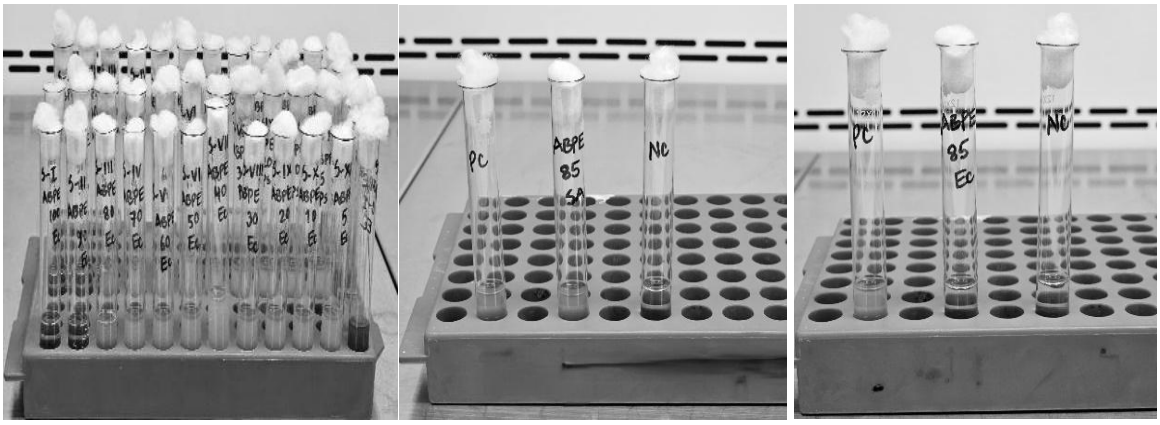


Figure 3: Determination of MIC of ABPE against *S. aureus* and *E. coli*

Table III: Antibacterial activity of different concentrations of ABPE measured in Zone of Inhibition

| Concentrations of ABPE solutions in each disc mg/ml | Zone of inhibition (ZOI) in mm | |
|---|---|---|
| | *Staphylococcus aureus* | *Escherichia coli* |
| 100 | 18 | 16 |
| 80 | 06 | 06 |
| 60 | 06 | 06 |
| 40 | 06 | 06 |
| 20 | 06 | 06 |
| 10 | 06 | 06 |
| 05 | 06 | 06 |
| Negative control | 06 | 06 |

Table IV: Bacterial zone of inhibition at different concentrations of ABPE for repeat experiment

| Test Organism | Concentration of ABPE (mg/ml) | ZOI (in mm) |
|---|---|---|
| *Staphylococcus aureus* | 90 | 11 |
| *Escherichia coli* | 90 | 12 |

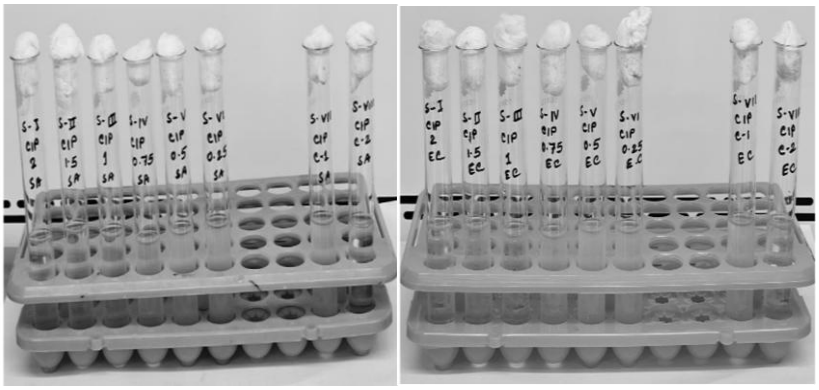

Figure 4: Determination of MIC of Ciprofloxacin

**Discussion**

Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* were evaluated against an aqueous seeds extract of black pepper. The Kirby-Bauer disc diffusion technique was used to evaluate the antibacterial activity as millimeter-sized zones of growth inhibition. The MIC was defined as the lowest amount of extract necessary to stop test organisms from growing visibly. After comparing test organism growth to that of controls and adjusting for turbidity in the broth dilution procedure, visible growth of the test organisms was discovered. Clear solutions were seen as having "No growth", whereas turbid ones had "Growth" of germs. Black pepper seed extract in methanol shown strong anti-test-organism activity. Effective Zone of Inhibition (ZOI) of ABPE for *Staphylococcus aureus* was 11 mm at 90 mg/ml concentration and maximum ZOI was 18 mm at 100 mg/ml concentration. For *Escherichia coli* effective Zone of Inhibition (ZOI) also started from 90 mg/ml concentration (12 mm) and at 100 mg/ml concentration maximum ZOI was 16 mm. In this study, for ABPE at 100 mg/ml concentration the ZOI was 18 mm against *S. aureus*, for *E. coli* it was 16 mm. Antibacterial activities of Aqueous and organic seed extracts of Black pepper was assessed by using agar disc diffusion method against Gram-positive and Gram-negative bacteria by Gupta et al.[12]. In Aqueous extract ZOI for *Staphylococcus aureus* was 7 mm and *Escherichia coli* was 8 mm at 50 mg/ml concentration of the extract respectively, which is similar with present study[12].

Penecilla and Magno in 2011 examined the antibacterial activity of aqueous extracts of Black pepper by using agar disc diffusion method against Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa. In Aqueous extract ZOI for *Staphylococcus aureus* was 10 mm and for *Pseudomonas aeruginosa* was 13 mm at 1 mg/ml concentration of the extract respectively. The result is dissimilar with the present study. This dissimilarity may be due to the difference in the concentration of extract[13]. In this study, the MIC of ABPE for *S. aureus* 90 mg/ml and for *E. coli* MIC was 85 mg/ml concentration. MIC of Aqueous seed extracts of Black pepper was assessed by Gupta et al. using broth dilution method against Gram-positive and Gram-negative bacteria. In Aqueous extract, the MIC for *S. aureus* was 50 mg/ml, and for *E. coli* MIC was 40 mg/ml. The result is dissimilar with the present study. This dissimilarity may be due to the difference in the concentration of extract[12]. MIC of *E. coli* was lower than the MIC of *S. aureus*. This may be caused by structural differences in the cell wall and membrane, since gram negative bacteria have an outer membrane that further prevents the entry of antibiotics, including seed extract, resulting in their resistance.

**Conclusion**

From the study it is clearly evident that aqueous extracts of Black pepper *(Piper nigrum L.)* seeds have dose dependent inhibitory effect against *Staphylococcus aureus* and *Escherichia coli*. Relatively higher concentrations of the extracts showed higher degrees of inhibition against the test organisms. Further studies are required to detect and isolate the biologically active ingredients present in the Black pepper which are responsible for this antibacterial effect.

**References**


1. Butler MS, Buss AD. Natural products- the future scaffolds for novel antibiotics? Biochemical Pharmacology. 2006;71(7):919-29.
2. Martens E, Demain AL. The antibiotic resistance crisis, with a focus on the United States. The Journal of Antibiotics. 2017;70(5): 520-6.
3. Dadgostar P. Antimicrobial resistance: implications and costs. Infection and Drug Resistance. 2019:3903-10.
4. Jamshidi-Kia F, Lorigooini Z, Amini-Khoei H. Medicinal plants: Past history and future perspective. Journal of Herbmed Pharmacology. 2017;7(1):1-7.
5. Aldaly ZT. Antimicrobial activity of piperine purified from Piper nigrum. J Basrah Res. 2010;36:54-61.
6. Shaheen N, Akhter-Sultan R, Abidi S, Azhar I, Mahmood ZA. Pharmacognostic evaluation and instrumental analysis (sem) for the standardization of Piper nigrum L. (black pepper) fruit. Pak J Bot. 2019;51:1859-63.
7. Kaho ZM, Kadum AR, Hadi AA. Evaluation of antibacterial activity of Piper nigrum extract against Streptococcus mutans and Escherichia coli. Journal of Pharmaceutical Sciences and Research. 2019;11(2):367-70.
8. Damanhouri ZA, Ahmad A. A review on therapeutic potential of Piper nigrum L. Black Pepper): The King of Spices. Med Aromat Plants. 2014;3(3):161.
9. Shaikh JR, Patil MK. Recent Advances in Pharmacology of Piper nigrum and Piperine: A Review. International Journal of Innovative Science Engineering and Technology (IJISET).
10. Ali M, SS A, Nas FF. Phytochemistry and antibacterial activity of black pepper (Piper nigrum) seeds extracts on some food borne pathogens. Inte Jr Pharmco Scie An Chem: IJPSC-105. 2018.
11. Alves TM, Silva AF, Brandão M, Grandi TS, Smânia ED, Smânia Junior A, Zani CL. Biological screening of Brazilian medicinal plants. Memórias do Instituto Oswaldo Cruz. 2000;95:367-73.
12. Gupta N, Parashar P, Mittal M, Mehra V, Khatri M, Rajguru S. Antibacterial potential of Elletaria cardamomum, Syzygium aromaticum and Piper nigrum, their synergistic effects and phytochemical determination. J Pharm Res. 2014;8(8):1-7.
13. Penecilla GL, Magno CP. Antibacterial activity of extracts of twelve common medicinal plants from the Philippines. Journal of Medicinal Plants Research. 2011;5(16): 3975-81.