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PHYTOCHEMICAL ANALYSIS, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY DETERMINATION OF *PIPER NIGRUM*

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ABSTRACT

To evaluate the phytochemical constituents, antibacterial and antioxidant scavenging activity of ethanolic Soxhlet and maceration extract of *Piper nigrum* fruit. In the present research, Soxhlet extraction and maceration extraction of *Piper nigrum* were carried out by using 95% ethanol as solvent. Phytochemical analysis of the important chemical constituents were carried out for both the ethanolic extracts. Antibacterial activity of *Piper nigrum* extract was determined by using well diffusion method and the zone of inhibition was measured. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were carried out subsequently. Antioxidant activity of *Piper nigrum* was determined by using DPPH radical scavenging assay and the IC_{50} values were determined. Total phenolic content was determined by the Folin-Ciocalteu method and expressed as mg Gallic acid per gram of plant material. For phytochemical analysis, both extracts showed positive results in all the tests except in the test for saponin, anthraquinone, glycoside and flavonoid. For the well diffusion test, positive results were seen in all the bacterial strains except for *Escherichia coli*. The MIC and MBC could not be determined accurately as the concentration used was too low. For antioxidant analysis, DPPH radical scavenging activity and total phenolic contents showed a positive correlation whereby the activity increases with the concentration. It is concluded that *Piper nigrum* exhibits antibacterial and antioxidant properties. It is concluded that *Piper nigrum* exhibits antibacterial and antioxidant properties.

KEYWORDS: *Piper nigrum*, maceration, Soxhelt, antibacterial, antioxidant.

INTRODUCTION

Piper nigrum (black pepper), known as the 'King of Spices' is considered as one of the most valuable medicinal plant. It belongs to the pepper family otherwise known as Piperaceae. It is native to southern India and Sri Lanka and in many tropical regions like Brazil and Indonesia.

Piper nigrum is widely used as seasoning in traditional cuisines, as food preservative, perfumery and in the Indian and Chinese traditional medicines. The fruits have a bitter, hot, sharp taste and tend to produce salivation and numbness of the mouth.[2] *Piper nigrum* has a distinct aroma due to its essential oil, while its characteristic pungency is mainly due to its alkaloid constituent piperine.[3] Ever since the active ingredient piperine was discovered, the use of black pepper has caught the interest of many modern medical researchers. Piperine has been shown to be responsible for the many pharmacological actions of black pepper such as antioxidant, antipyretic, analgesic, antihypertensive, antiplatelet, anti-inflammatory, anti- asthmatic, anti-spasmodic, anti-diarrheal, antibacterial, antifungal, antimutagenic and antitumor.[1] The fruit and plant are shown in Figures 1a – 1d, respectively.[4,5,10]

Figure 1a: Fruits of *Piper nigrum*.[4]

Figure 1b: *Piper nigrum* leaves.[5]

Figure 1c: *Piper nigrum* leaves with unripe green fruits.[5]

Figure 1d: *Piper nigrum* plantation.[10]

Phytochemical screening of *Piper nigrum* fruit extracts (water, methanol, and ethanol) revealed the presence of a range of secondary metabolites, including alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and anthraquinones.[14] Many other compounds which were also identified which include brachyamide B, dihydro-pipericide, (2E,4E)-N- eicosadienoylpereridine, N-trans-feruloyltryamine, N-formyl-piperidine, guineensine, pentadienoyl as piperidine, (2E,4E)- N-isobutyl-idecadienamide, isobutyl-eicosadienamide, tricholein, trichostachine, isobutyl-eicosatrienamide, isobutyl-octadienamide, piperamide, piperamine, piperettine, pipericide, piperine, piperolein B, sarmentine, sarmentosine and retrofractamide A. The different pharmacological activities were reported due to the presence these phytochemicals.[15] Piperine is a naturally found alkaloid produced as secondary metabolite in plants belonging to the pyridine group of Piperaceae family. Piperine is the trans-stereoisomer of 1-piperoylpiperidine. It is also known as (E, E)-1-piperoylpiperidine and (E, E)-1- [5-(1, 3 benzodioxol-5-yl)-1-oxo-2, 4-pentdienyl] piperidine. It was reported to have four isomers: piperine, isopiperine, chavicine and isochavicine.[6] It has also been used as an insecticide and herbal cough syrups. Recent medical studies have shown that it is helpful in increasing the absorption of certain vitamins, selenium, β-cartene, also increase the body's natural thermogenic activity.[16-24]

Experimental Methodology

Extraction of plant fruit material.[25-40]

Soxhlet extraction of *Piper nigrum* with ethanol 95%

500 mg of the powdered sample was added with 500 ml of ethanol 95% in the Soxhelt apparatus and heated at 65°C. The cycle was repeated until the solvent in the flask is enriched with the extracted material.[25] The extract in the round bottom flask was collected, measured and evaporated by using the rotary evaporator at a temperature of 60 °C at 100 rotations per minute (rpm) to a volume of 50 ml. The concentrated extract was transferred to a porcelain dish and further dried by using the water bath at a temperature of 70 °C until it becomes semi-solid (gummy mass). The porcelain dish was then sealed with aluminium foil and the gummy Soxhlet extract was kept in the refrigerator until further use.

Maceration extraction of *Piper nigrum* with ethanol 95%

200 gm of black pepper powder was weighed and with 400 ml of extracting solvent which is the 95% ethanol. The flask was consistently shaken 3 times a day over a period of 7 days. The extract was filtered from the residues by using two clean muslin cloths. The filtered extract was collected, measured and evaporated by using the rotary evaporator at a temperature of 60 °C at 100 rotations per minute (rpm) to a volume of 50 ml. The concentrated extract was transferred to a porcelain dish and further dried by using the water bath at a temperature of 70 °C until it becomes semi-solid (gummy mass). The porcelain dish was then sealed with aluminium foil and the gummy maceration extract was kept in the refrigerator until further use.

Phytochemical screening tests

In order to evaluate the phytochemistry of *Piper nigrum*, both the ethanolic Soxhlet and maceration extracts were subjected to various preliminary tests to determine the

presence of alkaloids, reducing sugars, saponins, terpenoids or steroids, anthraquinones, glycosides, tannin, flavonoids, carbohydrate and phenols. The results of the tests were observed and recorded accordingly. Table 1 indicates the phytochemical results obtained.

Antimicrobial activity screening

1. Nutrient agar (HiMedia M001- 100G).
2. Nutrient broth (HiMedia M002-100G).
3. Dimethyl sulfoxide (DMSO) solution (Fisher Scientific UK).
4. Double Distilled water freshly prepared in the laboratory.
5. 70% ethanol freshlydistilled in the laboratory.

Bacterial strains

- *Bacillus subtilis* (ATCC 6633)
- *Staphylococcus aureus* (ATCC 29737)
- *Escherichia coli* (ATCC 8739)
- *Pseudomonas aeruginosa* (ATCC 27853)

Well diffusion test

The well diffusion test was employed to find out the antimicrobial activity of *Piper nigrum* against four different bacterial strains- Gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus)* and Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The nutrient agar was used as the medium for the growth of the mentioned bacteria. The ethanolic maceration extract of *Piper nigrum* was used in this test. Aseptic techniques were practiced in which all the steps were carried out in the laminar airflow cabinet.

Dilution of extracts

Six different concentrations of the ethanolic maceration extract were prepared at 100, 80, 60, 40, 20 10 mg/ml using the serial dilution method. A stock solution of the extract with 100 mg/ml concentration was first prepared by dissolving 4000 mg of the dried gummy extract in 40 ml of pure dimethyl sulfoxide (DMSO) solution. From the stock solution, 10, 8, 6, 4, 2, 1 ml were each pipetted into 6 different tubes which had been labelled accordingly. The volume of each tube was then made up to 10 ml using DMSO solution. To prepare 100 mg/ml concentration, 10 ml was taken directly from the stock solution. To prepare 80 mg/ml concentration, 8 ml was taken from the stock solution and diluted with 2 ml of DMSO solution. To prepare 60 mg/ml concentration, 6 ml was taken from the stock solution and diluted with 4 ml of DMSO solution. To prepare 40 mg/ml concentration, 4 ml was taken from the stock solution and diluted with 6 ml of DMSO solution. To prepare 20 mg/ml concentration, 2 ml was taken from the stock solution and diluted with 8 ml of DMSO solution. To prepare 10 mg/ml concentration, 1 ml was taken from the stock solution and diluted with 9 ml of DMSO solution.

Preparation of bacterial cultures and agar media

To culture the bacterial strains, nutrient broths were prepared by dissolving 1.4 g of nutrient broth powder in 100 ml distilled water and sent for autoclave. A colony of each bacterial strain (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa*) was separately and aseptically inoculated into the sterile nutrient broths using a bacteriological loop. The cultured broths were then incubated at 37°C for approximately 18 hours. As for the agar plate media, for each litre of nutrient agar required, 28 g of nutrient agar powder was dissolved in distilled water and sent for autoclave. Since the test was done in duplicate sets, 25 petri dishes were prepared and filled with nutrient agar. 16 plates were used for the spreading of the bacterial cultures (4 plates for the each of the 4 bacterial strains) followed by pouring of extract into the wells. The next 4 plates were used for positive control which were each spread with only the bacterial cultures without any wells made. Another 4 plates were used for DMSO control in which the agar plates were spread with bacterial cultures followed by addition of DMSO solution into the wells. 1 plate was kept aside for negative control without the bacterial inoculum.

Well diffusion test procedure

0.1 ml of the prepared bacterial culture (*Bacillus subtilis*) was inoculated onto the surface of the agar media using a micropipette and spread evenly with a sterile spreader. The diluted extract solutions with the concentration of 100, 80, 60 mg/ml were each poured into the wells, followed by 40, 20 and 10 mg/ml in another agar plate. The wells were filled up to approximately 70% of its height. The process was repeated for the other bacterial strains (*Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa*) and duplicate sets were made for all. All the plates were kept in the refrigerator for at least one hour to allow for diffusion of extract into the agar. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was evaluated after incubation by measuring the zone of inhibition in millimetres and the mean readings were taken.

Minimum inhibitory concentration (MIC)

The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate any visible growth. It was determined by the serial dilution method and the microorganism growth was indicated by turbidity. [28, 34] The inoculum of each bacterial strain (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*) was prepared and the suspensions were adjusted according to the 0.5 McFarland Standard with absorbance range of 0.08 to 0.10 at 625 nm.

Preparation of standard McFarland bacterial culture

4-5 loops of the bacterial strains were each cultured in sterile nutrient broth and incubated at 37°C for approximately 24 hours. After incubation, the cell mass was obtained by centrifuging the culture at 5000 rpm for 10 minutes. The supernatant was discarded and the resulting cell mass was then re-suspended in another new

sterile nutrient broth. The suspensions were standardized according to the McFarland turbidity standard using a spectrophotometer at 625 nm.

MIC serial dilution procedure

30 g of the dried gummy macerated extract was dissolved in 3 ml of DMSO solution to obtain a concentration of 1000 µg/ml. Two fold serial dilutions were made to obtain five different concentrations of 1000, 500, 250, 125 and 62.5µg/ml. To obtain the 1000 µg/ml concentration, 0.2 ml of the extract solution was transferred into a clean assay tube and added with 1.8 ml of the standard McFarland culture (Bacillus subtilis) which was previously prepared. To obtain the 500 µg/ml, 250 µg/ml, 125 µg/ml and 62.5 µg/ml concentrations, 1 ml of the mixture from the first tube was transferred to another tube and added with 1 ml of the standard McFarland culture, respectively. The entire process was repeated for the other bacterial strains (Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa) and duplicate sets were made for all. Four positive tubes containing only the bacterial culture suspension were prepared respectively. One negative tube containing only the sterilised nutrient broth was also prepared. The tubes were then incubated at 37°C for 24 hours. The MIC activity was evaluated by observing the turbidity of the tubes after incubation. The tube that does not show any visible turbidity indicates the MIC.

Minimum bactericidal concentration (MBC)

MBC is known as the lowest concentration of the extract at which the incubated microorganism was completely killed. [(11)] The tubes which did not show any visible turbidity from the MIC test were taken and proceeded with the MBC determination.

0.1 ml was taken from the tube and inoculated onto nutrient agar plates and the plates were then incubated at 37°C for 24 hours. The MBC activity was evaluated by observing the presence of bacterial growth on the agar after incubation. The plate that does not have presence of bacterial growth indicated the MBC.

Antioxidant activity screening DPPH radical scavenging assay

To evaluate the antioxidant activity of *Piper nigrum*, 1,1 Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was carried out. DPPH is a free radical which has a strong purple colour. When antioxidants react with DPPH, it is reduced to the DPPH-H form (non-radical) which is yellow in colour. The degree of decolourization to yellow indicates the scavenging efficiency of the extract. The assay is based on measurement of the loss of DPPH colour at 518 nm using UV-visible spectrophotometer. The lower the absorbance value, the better the antioxidant capacity of the extract.

Dilution of extracts and standards

The ethanolic Soxhlet extract was used in this assay. Six different concentrations of extracts were prepared at 10, 20, 40, 60, 80 and 100 µg/ml using serial dilution method. The stock solution was first prepared by dissolving 10 mg of extract in 10 ml of 95% ethanol. 1 ml of the solution was then diluted ten-fold with 9 ml of 95% ethanol to obtain a concentration of 100µg/ml. From the stock solution, 10, 8, 6, 4, 2, 1 ml were pipetted into 6 different tubes labelled accordingly. The volume was then made up to 10 ml using 95% ethanol. To prepare 100 µg/ml concentration, 10 ml was taken directly from the stock solution. To prepare 80 µg/ml concentration, 8 ml was taken from the stock solution and diluted with 2 ml of 95% ethanol solution. To prepare 60 µg/ml concentration, 6 ml was taken from the stock solution and diluted with 4 ml of 95% ethanol solution. To prepare 40 µg/ml concentration, 4 ml was taken from the stock solution and diluted with 6 ml of 95% ethanol solution. To prepare 20 µg/ml concentration, 2 ml was taken from the stock solution and diluted with 8 ml of 95% ethanol solution. To prepare 10 µg/ml concentration, 1 ml was taken from the stock solution and diluted with 9 ml of 95% ethanol solution. Butylated hydroxyltoluene (BHT) was used as standard in this assay. Six different concentrations of BHT solutions were prepared at 10, 20, 40, 60, 80 and 100 µg/ml.

DPPH assay procedure

0.1mM of ethanolic solution of DPPH was freshly prepared by dissolving 3.94 mg of DPPH crystalline powder in 100 ml of 95% ethanol and stored in amber coloured bottle. 3 ml of 0.1 mM DPPH reagent was added to 2.5 ml of the different concentrations of ethanolic extracts. A control containing only 3 ml of DPPH solution and 2.5 ml of ethanol was used. The mixtures were shaken and labelled accordingly and left to stand in the dark at room temperature for 30 minutes. Approximate amount of each mixture was then transferred into a cuvette and the absorbance was measured at 518 nm using a spectrophotometer. The same procedure was repeated by replacing the extracts with BHT solutions of different concentrations. The absorbance for both ethanolic extract and BHT solutions were tabulated and calibration curves were constructed. The antioxidant activity was determined by the following formula:

$$\text{DPPH Radical- scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where Acontrol and Asample are the absorbance of the control and of the sample respectively.[27] The 50% inhibitory concentration value (IC50) of the samples were calculated and it denotes the concentration of the sample that is required to scavenge 50% of the DPPH free radicals.

Total phenolic content

The total phenolic content was determined by using the Folin-Ciocalteu method. The absorbance is measured at

750nm. The higher the absorbance, the higher the total phenolic content. Since Gallic acid is used as the standard, it is also known as Gallic Acid Equivalent Method (GAE). The absorbance of the extract was compared with Gallic acid equivalents calibration curve. The total phenolic content of the extract was expressed as mg Gallic acid equivalents per gram of extract (mg GAE) / g).

Preparation of extract solution

The ethanolic Soxhlet extract was used in this assay. Four different concentration of extracts were prepared at 25, 50, 100 and 200 µg/ml using serial dilution method. The stock solution was first prepared by dissolving 20 mg of extract in 10 ml ethanol. 1 ml of the solution was then diluted ten-fold with 9 ml of ethanol to obtain a concentration of 200 µg/ml. From the stock solution, 2 ml was taken and double diluted with 2 ml ethanol to obtain a concentration of 100 µg/ml. From the 100 µg/ml concentration solution, 2 ml was taken and double diluted with 2 ml ethanol to obtain a concentration of 50 µg/ml. The same procedure was repeated by taking 2 ml from the 50 µg/ml concentration solution and dilute with 2 ml ethanol to obtain a concentration of 25 µg/ml.

Preparation of standard solution (Gallic acid)

Gallic acid was used as standard in this assay. Five different concentrations of Gallic acid were prepared at 1, 2, 4, 6, 8 and 10 µg/ml. The stock solution was first prepared by dissolving 10 mg of Gallic acid in 10 ml of 95% ethanol. 1 ml of the solution was then diluted ten-fold with 9 ml of 95% ethanol to obtain a concentration of 100 µg/ml. The same step is repeated until the final concentration of 10 µg/ml was obtained. From the stock solution, 10, 8, 6, 4, 2, 1 ml were pipetted into 6 different tubes labelled accordingly. The volume was then made up to 10 ml using 95% ethanol.

Total phenolic content procedure

For every 0.2 ml of extract, about 0.2 ml of Folin-Ciocalteau reagent and 4 ml of 2.5 % sodium carbonate were added. The mixture was completely shaken and allowed to stand for two hours. Then, the absorbance of the solution was measured using UV spectrophometer at 750 nm. The same procedure was repeated by replacing the extract with Gallic acid. Quantification of total phenolic content was done using standard curve of Gallic acid as a standard phenolic compound (1,2,4,6,8,10 µg/ml), which was dissolved in ethanol and expressed as mg Gallic acid per gram of plant material.

RESULTS

Table 1: Phytochemical analysis of ethanolic Soxhlet and Maceration extracts of *Piper nigrum*.

No.	Phytochemical	Name of Test	Soxhlet extract	Maceration extract
1	Alkaloid	Dragendoff's test	+	+
2	Reducing sugar	Fehling's test	+	+
3	Saponin	Frothing test	-	-
4	Terpenoid or steroid	Salkowski test	+	+
5	Anthraquinone	Borntrager's test	-	-
6	Glycosides	Glycoside test	-	-
7	Tannin	Ferric chloride test	+	+
8	Flavonoid	Sodium hydroxide test	-	-
9	Carbohydrate	Molisch's test	+	+
10	Phenol	Lead acetate test	+	+

+: present -: absent

Antimicrobial activity Well diffusion test

Well diffusion test was carried out using ethanolic maceration extract and the measurement of zone of inhibitions were tabulated. The results of zone of inhibition for *Bacillus subtilis* (set1 and 2) are shown in Tables 2 and 3, respectively.

Table 2: Zone of inhibition for *Bacillus subtilis* (Set 1).

Concentration (mg/ml)	Inhibition zone diameter readings (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	5	5	5	6	5	6	5	5	**5.25**
20	7	6	6	6	7	7	6	7	**6.50**
40	8	9	9	8	8	7	8	9	**8.25**
60	4	3	3	4	2	2	3	3	**3.00**
80	4	4	5	4	3	4	3	4	**3.89**
100	3	4	2	3	4	4	2	3	**3.13**

Table 3: Zone of inhibition for *Bacillus subtilis* (Set 2).

Concentration (mg/ml)	Inhibition zone diameter readings (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	2	3	3	4	3	3	4	3	**3.13**
20	3	2	3	3	5	4	4	5	**3.63**
40	5	3	3	5	4	5	4	4	**4.13**
60	2	3	2	3	2	2	3	2	**2.38**
80	3	3	2	3	2	3	3	2	**2.63**
100	2	2	2	3	2	3	3	3	**2.50**

The results of zone of inhibition for *Staphylococcus aureus* (set1 and 2) are tabulated in Table 4 and 5, respectively.

Table 4: Zone of inhibition for *Staphylococcus aureus* (Set 1).

Concentration (mg/ml)	Inhibition zone diameter readings (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	4	4	5	5	4	5	4	4	**4.38**
20	5	4	5	4	5	4	4	5	**4.50**
40	6	6	7	5	6	7	5	5	**5.89**
60	7	6	8	7	6	7	8	6	**6.88**
80	6	8	7	7	8	6	8	8	**7.25**
100	7	8	8	7	8	8	8	7	**7.63**

Table 5: Zone of inhibition for *Staphylococcus aureus* (Set 2)

Concentration (mg/ml)	Inhibition zone diameter readings (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	3	3	3	4	3	4	3	3	**3.25**
20	3	3	4	4	3	4	3	3	**3.38**
40	4	4	5	4	4	5	5	4	**4.38**
60	6	6	7	6	7	8	8	6	**6.75**
80	7	7	6	8	6	7	8	8	**7.13**
100	8	8	7	8	8	7	7	7	**7.50**

The results of zone of inhibition for *Escherichia coli* (set1 and set2) are shown in Table 6 and 7 respectively.

Table 6: Zone of inhibition for *Escherichia coli* (Set 1).

Concentration (mg/ml)	Inhibition zone diameter readings (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-

Table 7: Zone of inhibition for *Escherichia coli* (Set 2)

Concentration (mg/ml)	Inhibition zone diameter readings (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-

The results of zone of inhibition for *Pseudomonas aeruginosa* (set1 and 2) are placed in Table 8 and 9, respectively.

Table 8: Zone of inhibition for *Pseudomonas aeruginosa* (Set 1).

Concentration (mg/ml)	Inhibition zone diameter readings (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	2	3	3	2	3	3	2	3	**2.63**
20	5	4	4	5	5	4	5	4	**4.50**
40	5	6	6	5	6	6	5	6	**5.63**
60	7	6	7	7	6	6	7	7	**6.63**
80	6	5	5	6	5	5	5	5	**5.25**
100	6	5	6	6	6	5	6	5	**5.63**

Table 9: Zone of inhibition for *Pseudomonas aeruginosa* (Set 2).

Concentration (mg/ml)	Inhibition zone diameter readings (mm)								
	1st	2nd	3rd	4th	5th	6th	7th	8th	Mean
10	3	2	3	3	3	2	2	3	**2.63**
20	3	3	3	2	3	3	2	3	**2.75**
40	5	4	4	4	5	3	3	3	**3.89**
60	5	7	7	6	6	5	7	6	**6.13**
80	6	5	5	7	7	5	6	6	**5.88**
100	7	6	6	7	6	6	7	6	**6.38**

The Agar plate results for *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa are shown in Figures 3a – 3l.*

1. **Agar plate results with *Bacillus subtilis***

Figure 3a: DMSO control plate and positive control plate of *Bacillus subtilis.*

Figure 3b: *Piper nigrum* extract with *Bacillus subtilis* at 10, 20, 40 mg/ml concentration.

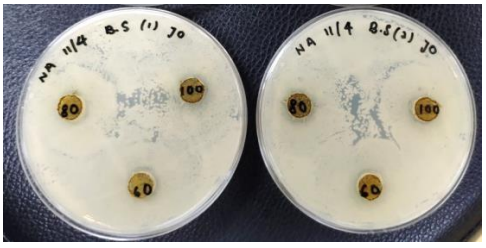


Figure 3c: *Piper nigrum* extract with *Bacillus subtilis* at 60, 80, 100 mg/ml concentration.

2. **Agar plate results for *Staphylococcus aureus***

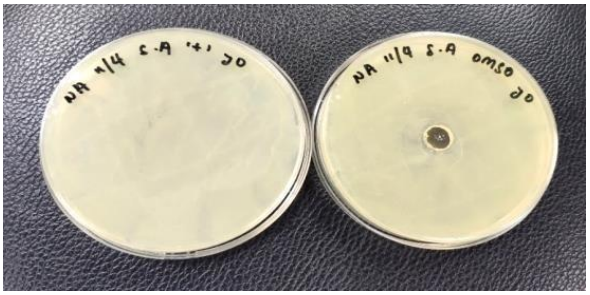


Figure 3d: DMSO control plate and positive *Staphylococcus aureus.*

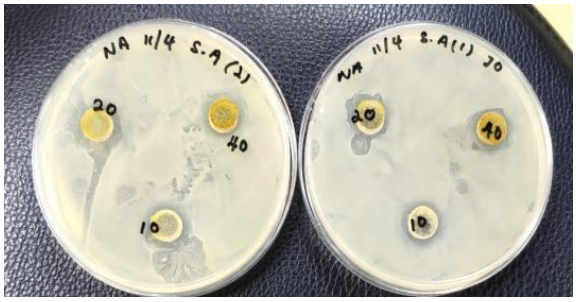


Figure 3e: *Piper nigrum* extract with control plate of *Staphylococcus aureus* at 10, 20, 40 mg/ml concentration.

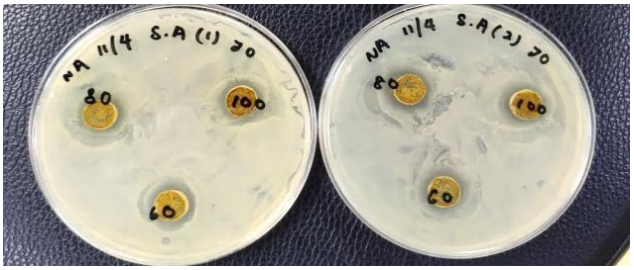


Figure 3f: *Piper nigrum* extract with *Staphylococcus aureus* at 60, 80, 100 mg/ml concentration.

3. **Agar plate results for *Escherichia coli***

Figure 3g: DMSO control plate and positive plate of *Escherichia coli.*

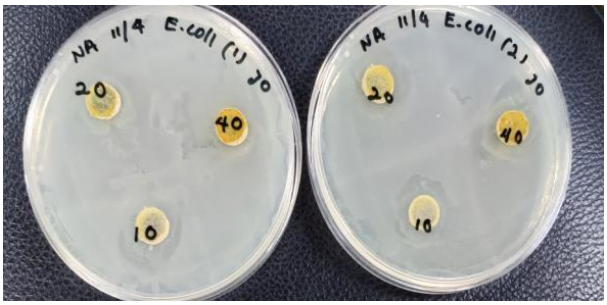


Figure 3h: *Piper nigrum* extract with control *Escherichia coli* at 10, 20, 40 mg/ml concentration.

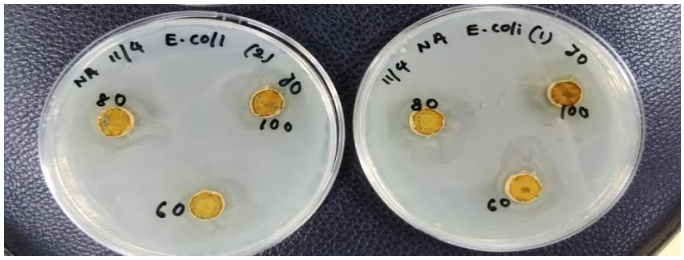


Figure 3i: *Piper nigrum* extract with *Escherichia coli* at 60, 80, 100 mg/ml concentration.

4. **Agar plate results for *Pseudomonas aeruginosa***

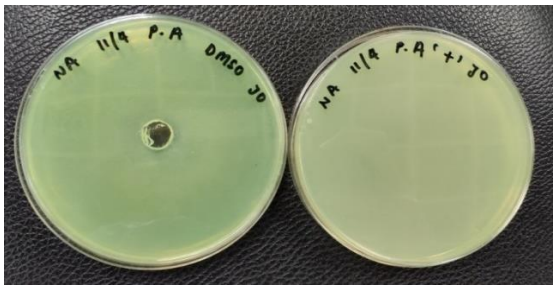


Figure 3j: DMSO control plate and positive of *Pseudomonas aeruginosa.*

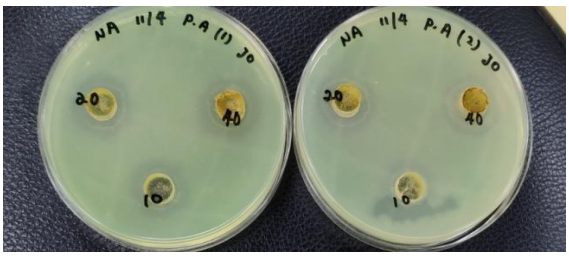


Figure 3k: *Piper nigrum* extract with control plate *Pseudomonas aeruginosa* at 10, 20, 40 mg/ml concentration.

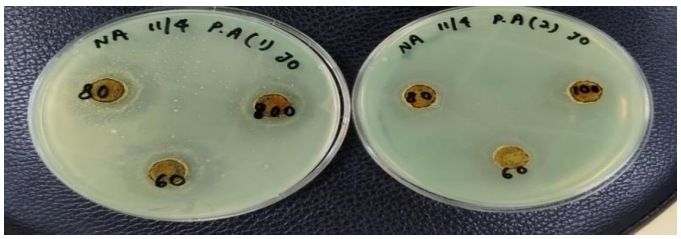


Figure 3l: *Piper nigrum* extract with *Pseudomonas aeruginosa* at 60, 80, 100 mg/ml concentration.

Minimum inhibitory concentration (MIC)

After incubation, the tubes were seen to be turbid and bacterial growth were present in most of the tubes. Only the tubes with 1000 µg/ml and 500 µg/ml concentration for each bacterial strain were further tested for its minimum bactericidal concentration (MBC).

Minimum bactericidal concentration (MBC)

Minimum bactericidal concentration (MBC) results are shown in Figures 4a – 4d.

Results for *Bacillus subtilis* bacterial strain

Figure 4a: Results of MBC test for *Bacillus subtilis*.

1. **Results for *Staphylococcus aureus* bacterial strain**

Figure 4b: Results of MBC test for *Staphylococcus aureus.*

2. **Results for *Escherichia coli* bacterial strain**

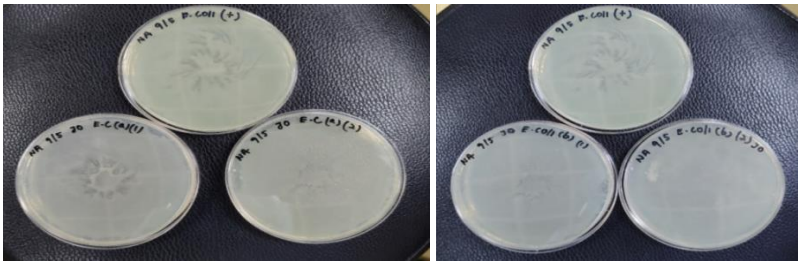


Figure 4c: Results of MBC test for *Escherichia coli.*

3. **Results for *Pseudomonas aeruginosa* bacterial strain**

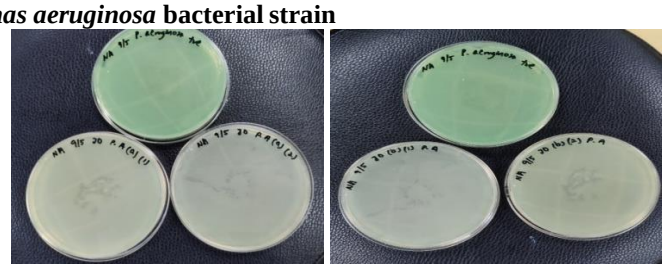


Figure 4d: Results of MBC test for *Pseudomonas aeruginosa*

Antioxidant activity

DPPH radical scavenging assay

The following results show the absorbance and corresponding percentage scavenging values of BHT standard solution and ethanolic Soxhlet extract of *Piper nigrum*.

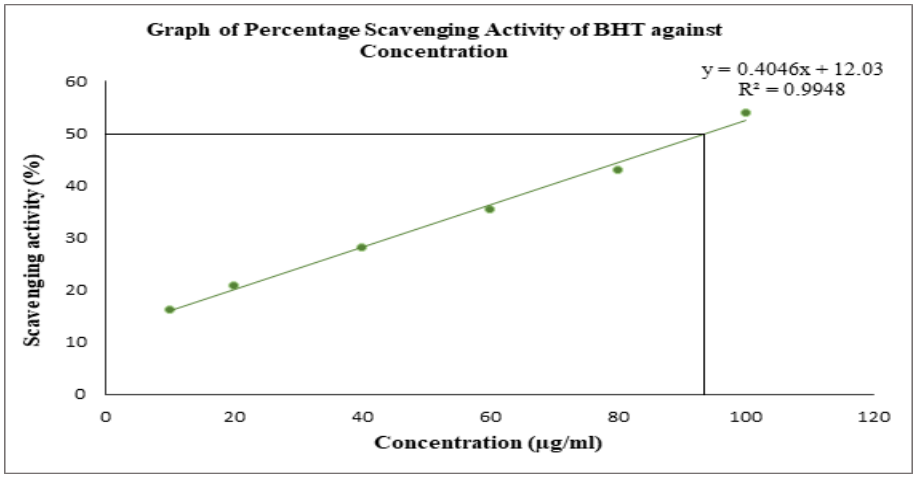
The control absorbance was measured at 0.582. Absorbance values and corresponding scavenging activity (%) of BHT and absorbance values and corresponding scavenging activity (%) of *Piper nigrum* is shown in Table 10 and 11 while the percentage scavenging activity of BHT against concentration and percentage Scavenging activity of *Piper nigrum* against concentration are shown in Graphs 1 and 2, respectively. The IC50 of the BHT standard solution was found to be 93.85µg/ml.

Table 10: Absorbance values and corresponding scavenging activity (%) of BHT.

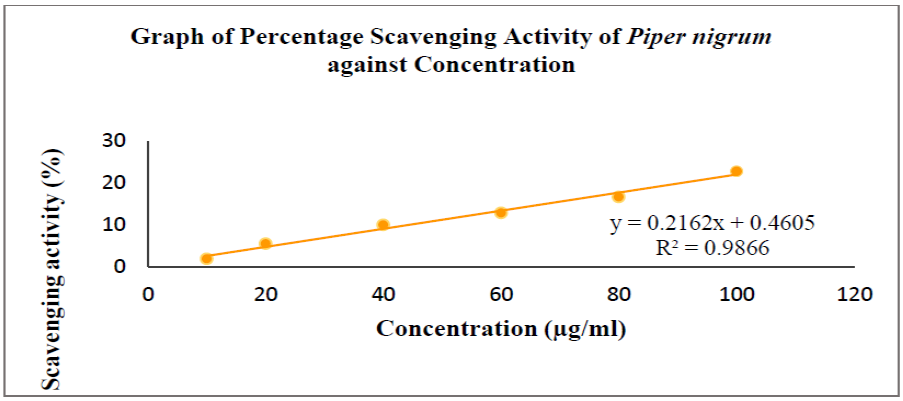
Conc. (µg/ml) of BHT standard solution	UV Absorbance	Scavenging activity (%)
10	0.488	16.15
20	0.461	20.79
40	0.418	28.18
60	0.375	35.57
80	0.332	42.96
100	0.268	53.95

Table 11: Absorbance values and corresponding scavenging activity (%) of Piper nigrum.

Conc. (µg/ml) of *Piper nigrum*	UV Absorbance	Scavenging activity (%)
10	0.571	1.89
20	0.550	5.50
40	0.524	9.97
60	0.507	12.89
80	0.485	16.67
100	0.449	22.85



Graph 1: Percentage Scavenging Activity of BHT against concentration.

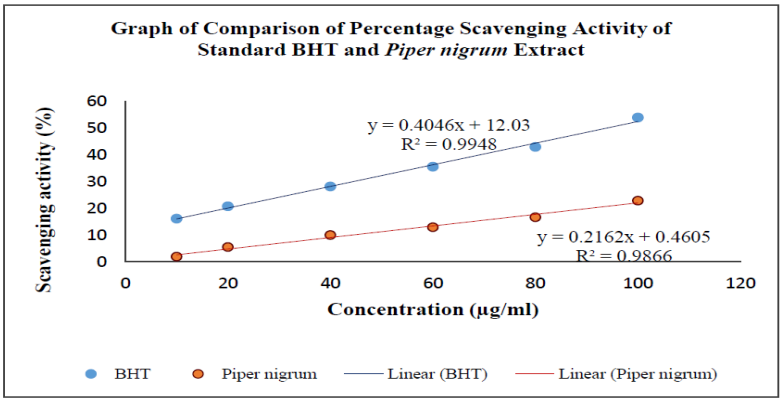


Graph 2: Percentage Scavenging Activity of *Piper nigrum* against concentration.

The comparison of scavenging activity (%) of BHT and *Piper nigrum* is shown in Table 12 and drawn in Graph 3.

Table 12: Comparison of scavenging activity (%) of BHT and *Piper nigrum*

Conc. (µg/ml)	% Scavenging	
	BHT	Piper nigrum
10	16.15	1.89
20	20.79	5.50
40	28.18	9.97
60	35.57	12.89
80	42.96	16.67
100	53.95	22.85



Graph 3: Comparison of Percentage Scavenging Activity of BHT and *Piper nigrum* against Concentration.

Total phenolic content determination

The following results show the absorbance values of Gallic acid standard solution and ethanolic Soxhlet extract of *Piper nigrum* as demonstrated in Table 13 and 14, while the calibration curve is shown in Graph 4.The concentration of total phenolic content in the extracts was calculated using the following equation:

C = ^{A} x Dilution factor $_{B}$

Where,

C: amount of total phenolic content

A = concentration of Gallic acid present (mg/ml) B: concentration of sample extracts

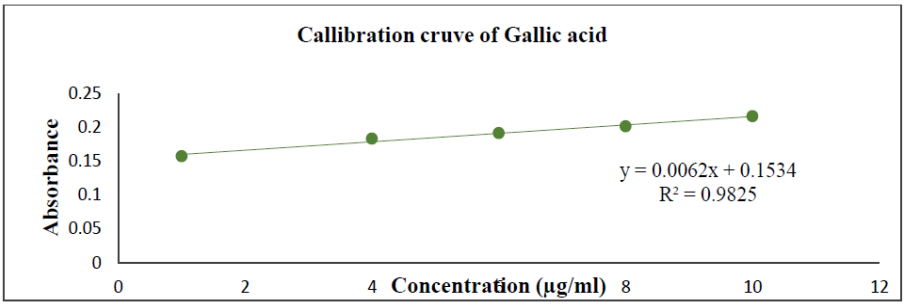
The total phenolic content of the sample extract is expressed as amount of Gallic acid equivalent per gram plant extract (mg GAE/g)

Table 13: Absorbance values of Gallic acid standard solution.

Concentration (µg/ml)	Absorbance
1	0.157
2	0.176
4	0.183
6	0.191
8	0.201
10	0.216

Table 14: Absorbance values of ethanolic Soxhlet extract of *Piper nigrum*.

Concentration (µg/ml)	Absorbance
25	0.206
50	0.242
100	0.325
200	0.481



Graph 4: Callibration curve of Gallic acid.

DISCUSSION

The phytochemical screening for both the ethanolic Soxhlet and maceration extracts of *Piper nigrum* were tabulated in Table 4.1. Both showed equal positive results in the test for alkaloid, reducing sugars, terpenoid, tannin, carbohydrates and phenol. Both of the extracts showed negative results in the test for saponin, anthraquinone, glycosides and flavonoids. From this, it can be deduced that the Soxhlet and maceration extraction methods have equal efficacy in extracting the same bioactive compounds. As the solvent used was 95% ethanol, some phytochemical constituents could have not been extracted completely or present in very minute amount, thus resulting in negative results. The tests conducted in this research were just preliminary screening. There are many more phytochemicals present in *Piper nigrum* reported in other studies. Extraction using other solvents such as methanol, chloroform, petroleum ether, acetone and distilled water showed varying proportions of phytochemicals.[26,38] In any research, it is necessary to choose the solvent according to its biological activity required and not the one which gives a high amount of bioactive compounds.[39]

The antibacterial activity of *Piper nigrum* was evaluated against four different bacterial strains- Gram (+) bacteria: *Bacillus subtilis* and *Staphylococcus aureus*, Gram (-) bacteria: *Escherichia coli* and *Pseudomonas aeruginosa*.

The zone of inhibition was clear, circular region, surrounding the wells present in the inoculated agar whereby there is no bacterial growth. Although not very significant, zone of inhibition were present in all of the plates inoculated with *B. subtilis*, *S. aureus* and *P. aeruginosa* except the plates inoculated with *E. coli*. This could be due to the extract concentration used was not high enough to exhibit any activity against the bacterial strain as other studies have proven the susceptibility of *Escherichia coli* towards the ethanolic extract *Piper nigrum*.[3,32,35] The test was conducted in duplicate sets to

obtain a more accurate results. However, the results obtained from both Set 1 and Set 2 did not correspond to each other. The results were not replicated accordingly. The rationale for this occurrence could be due to the bacterial cultures were not standardized according to the Mcfarland turbidity standards. Different densities of inoculum have been inoculated on the agar plates whereby certain plates showed a more abundant bacterial growth compared to the other plates. Due to this disproportionate of bacterial inoculum, lower concentration of extract showed better activity than the higher concentration extracts.

Depending on the tested concentration and different solvents used for *Piper nigrum* extraction, the susceptibility of the microorganisms was found to vary among studies. Among hexane, dichloromethane, ethanol, and aqueous extracts of *Piper nigrum* the dichloromethane extract displayed the highest activity against the bacteria *S. aureus, E. coli, S. typhi, and B. subtilis*.[47] Another study also found that n-Hexane solvent displayed no inhibition against *S. aureus, B. subtilis, E. coli*, and *P. aeruginosa*. In contrast, the acetone and ethanol extract of *Piper nigrum* were able to inhibit *S. aureus and B. subtilis.* [48]

For the minimum inhibitory concentration test, the same bacterial strains were used. The bacterial cultures were standardized according to the 0.5 Mcfarland turbidity standard with the absorbance range of 0.08 to 0.1 at 625 nm. This is to ensure that all the tubes are inoculated with similar densities of inoculum.

The extract concentration of 1000, 500, 250, 125 and 62.5µg/ml were used. Two fold serial dilution of the extract was carried out in the nutrient broth. After inoculation of the bacteria and incubation for 24 hrs, the tubes were examined for presence of turbidity. It has found that most of the tubes were turbid with presence of bacterial growth and residue like suspension as shown in Figure 4.21. The tubes with the least turbidity (tubes with concentration of 1000 µg/ml and 500 µg/ml) were chosen and proceeded with the MBC test to further confirm the presence/absence of bacterial growth.

For the minimum bactericidal concentration test, the bacterial suspension from the tubes chosen from MIC were cultured on nutrient agar plates. After incubation, the plates were observed for any bacterial growth in comparison with a positive control plate. *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed positive results in which no growth were present. Growth of *P. aeruginosa* was usually indicated with a green sheen and the absence of that in the MBC test shows that the MIC could be approximately around 500 µg/ml. Growth was seen in the plates inoculated with *Bacillus subtilis* and *Escherichia coli*.

The percentage scavenging of each concentration of BHT standard solution and extracts were calculated. From the results, it was deduced that the scavenging activities were concentration dependent. Highest percentage scavenging activity53.95% and 22.85 % were seen at the highest concentration of 100 µg/ml for BHT and ethanolic Soxhlet extract of *Piper nigrum* respectively. All the results of the extract were significantly lower than the standard BHT solution. *Piper nigrum* has long been documented to possess excellent antioxidant activities as it contains significant source of phenolic and flavonoid antioxidants.[14,25,27,50] The failure of reproducibility of strong antioxidant activity could be due to low concentration of extract used or due to failure of extraction of flavonoids in the ethanol solvent. Therefore, the lower the concentration needed to scavenge 50% of the DPPH free radicals, the better the antioxidant activity.

IC_{50} was not calculated in the case of the *Piper nigrum* extract as the highest percentage scavenging activity was found to be only 22.85% at 100µg/ml. This is because the determination of IC_{50} requires interpolation within the range of the data points. Since the reading did not exceed 50%, therefore the graph plotted cannot be used to determine the IC_{50}. In a study carried out by (Gayatri, 2011), the IC_{50} of ethanolic extract of *Piper nigrum* was found to be 14.15±0.02 µg/ml. (14) In another study conducted by (Nooman et al., 2008), the IC_{50} of crude methanolic extract of *Piper nigrum* was found to be 144.1 ± 2.2µg/ml.[37]

The results of the absorbance values of Gallic acid standard solution and ethanolic Soxhlet extract of *Piper nigrum* were tabulated. The extract showed an increase in anti-oxidant activity as the concentration increases. However, the results were significantly lower than the positive control gallic acid. A calibration curve of Gallic acid was plotted with an equation of y = 0.0062x + 0.1534. By substituting the absorbance value as y, the concentration of Gallic acid present (mg/ml) was determined. In a study conducted by (Gülçin, İ., 2005) 42.8 mg Gallic acid equivalent of phenols was detected in 1 mg of ethanol extract of black pepper.[25] Likewise, TPC value of 27 ±3 mg GAE/g extract of *Piper nigrum* was determined in another study.[3] Therefore, in comparison with these other studies, the total phenolic content obtained in this present study is relatively low. This may be associated to the low amount and incomplete solubilisation of phenolic compounds in the extract.

CONCLUSION

Phytochemical screening of the extracts revealed the presence of alkaloids, reducing sugar, terpenoids, tannin, carbohydrates, and phenol in the fruits. However, anthraquinones, glycosides and flavonoids could not be detected although these were proven to be present in other studies. The presence or absence of any one such phytochemical has a huge impact on its therapeutic properties. The antibacterial activity of the plant extract was determined by well diffusion method. Proper

standardization of bacterial inoculum is required to obtain better results. Different solvents used for extraction can affect the susceptibility of the microorganisms as well. DPPH assay was employed to evaluate the antioxidant activity of the extract while the total phenolic content was determined by Folin-Ciocalteu method. Both showed positive correlation whereby the antioxidant activity and the total phenolic content increased with the increase of concentration. The concentration of the extract used in the determination of antioxidant activity should be increased for better results. A more extensive research project should be further conducted to evaluate the other pharmacological uses of the plant. Usage of other solvents and extraction method to isolate more pure and higher yield of chemical constituents from the plants is recommended as well.

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REFERENCES

1. Damanhouri ZA. A Review on Therapeutic Potential of Piper nigrum L. (Black Pepper): The King of Spices. Med Aromat Plants [Internet], 2014; 03(03).
2. Ganesh P, Suresh Kumar R, Saranraj P. Phytochemical analysis and antibacterial activity of Pepper (Piper nigrum L.) against some human pathogens. Cent Eur J Exp Biol [Internet], 2014; 3(2): 36–41.
3. Andrade KS, Ferreira SRS. Antioxidant activity of black pepper (Piper nigrum L.) oil obtained by supercritical CO2. III Iberoam Conf Supercrit Fluids Cart indiad, 2013; 1–5.
4. Black pepper [Internet]. [cited 2019 Feb 10]. Available from: https://i.ebayimg.com/images/g/2y4AAOSwgv5ZR4Gz/s-l300.jpg
5. 220px-Piper_nigrum_-_Köhler–s_Medizinal-Pflanzen-107.jpg (220×281) [Internet]. [cited 2019 Feb 10].
6. Takooree H, Aumeeruddy MZ, Rengasamy KRR, Venugopala KN, Jeewon R, Zengin G, et al. A systematic review on black pepper (Piper nigrum L.): from folk uses to pharmacological applications. Crit Rev Food Sci Nutr [Internet], 2019; 0(0): 1–34.
7. Aggarwal BB, Kunnumakkara AB, Srinivasan K. Black Pepper (Piper nigrum) and Its Bioactive Compound, Piperine. Mol Targets Ther Uses Spices, 2010; 25–64.
8. Abdallah EM. Black pepper fruit (Piper nigrum L.) as antibacterial agent: A mini- review. J Bacteriol Mycol Open Access, 2018; 6(2): 141–5.
9. Black pepper spikes [Internet]. [cited 2019 Feb 10].
10. Piper nigrum plantation [Internet]. [cited 2019 Feb 10]. Available from: https://media-cdn.tripadvisor.com/media/daodao/photo-s/0d/3d/38/7d/this-is-the-first-place.jpg.
11. Shah CP, Santani D. Pharmacognostic , phytochemical analysis and antimicrobial activity of two piper species International Journal Of Comprehensive Pharmacy Pharmacognostic , Phytochemical Analysis And Antimicrobial Activity Of Two Piper Species, 2014; (May).
12. Hao C yun, Fan R, Ribeiro MC, Tan L he, Wu H song, Yang J feng, et al. Modeling the Potential Geographic Distribution of Black Pepper (Piper nigrum) in Asia Using GIS Tools. J Integr Agric, 2012.
13. Black Pipper: Sources, Cultivation and Uses (With Diagram) [Internet]. [cited 2019 Feb 10]. Available from: http://www.yourarticlelibrary.com/biology/plants/black- pipper-sources-cultivation-and-uses-with-diagram/49872
14. Nahak G, Sahu RK. Phytochemical evaluation and antioxidant activity of Piper cubeba and Piper nigrum. J Appl Pharm Sci., 2011; 1(8): 153–7.
15. Parmar VS, Jain SC, Bisht KS, Jain R, Taneja P, Jha A, et al. Phytochemistry of the genus Piper. Phytochemistry, 1997.
16. Use of piperine as a bioavailability enhancer., 1995 Oct 30. [cited 2019 Feb 23]; Available from: https://patents.google.com/patent/US5744161A/en.
17. Jagella T, Grosch W. Flavour and off-flavour compounds of black and white pepper (Piper nigrum L.) I. Evaluation of potent odorants of black pepper by dilution and concentration techniques. Eur Food Res Technol., 1999.
18. Joshi DR. A Review On Diversified Use Of The King Of Spices: Piper Nigrum (Black Pepper), 2018; 9(10): 4089–101.
19. Nadkarni KM. Indian Materia Medica With Ayurvedic, Unani-Tibbi, Siddka, .4ttopathic, Homeopathic, Naturopathic &- Home Remedies, Appendices & Indexes. Homeopathy, 2014.
20. Johri RK, Zutshi U. An Ayurvedic formulation "Trikatu" and its constituents. Journal of Ethnopharmacology. 1992.
21. Meghwal M, Goswami TK. Piper nigrum and piperine: An update. Phyther Res., 2013; 27(8): 1121–30.
22. Wang L, Zhao DY, Zhang ZH, Zuo CH, Zhang Y, Quan Pei Y, et al. Trial of antiepilepsirine (AES) in children with epilepsy. Brain Dev. 1999;
23. Doucette CD, Hilchie AL, Liwski R, Hoskin DW. Piperine, a dietary phytochemical, inhibits angiogenesis. J Nutr Biochem, 2013.
24. Makhov P, Golovine K, Canter D, Kutikov A, Simhan J, Corlew MM, et al. Co- administration of piperine and docetaxel results in improved anti-tumor efficacy via inhibition of CYP3A4 activity. Prostate, 2012.
25. Gülçin I. The antioxidant and radical scavenging activities of black pepper (Piper nigrum) seeds. Int J Food Sci Nutr., 2005; 56(7): 491–9.
26. Shailesh. Preliminary phytochemical and antimicrobial screening of Syzygium aromaticum, Elettaria cardamomum and Piper nigrum extracts. J Pharmacogn Phytochem, 2015; 4(3): 85–9.

27. Zarai Z, Boujelbene E, Ben Salem N, Gargouri Y, Sayari A. Antioxidant and antimicrobial activities of various solvent extracts, piperine and piperic acid from Piper nigrum. LWT - Food Sci Technol [Internet], 2013; 50(2): 634–41.
28. Karsha PV, Lakshmi OB. Antibacterial activity of black pepper (Piper nigrum Linn.) with special reference to its mode of action on bacteria. Indian J Nat Prod Resour, 2010;
29. Sharma PK, Baghel R, Vyas S, Ahirwar R. Phytochemical Investigation of Ethanolic Extract of Piper Nigrum, Zingiber Officinale, and Allium Sativum and Spectrophotometric Detection of Piperine, Gingerol, and Allicin. Int Res J Pharm, 2014; 5(11): 814–6.
30. Aldaly ZTK. Antimicrobial Activity of Piperine purified from piper nigrum PDF Created with deskPDF PDF Writer - Trial :: http://www.docudesk.com PDF Created with deskPDF PDF Writer - Trial :: http://www.docudesk.com, 2010; (5).
31. Rani SKS, Saxena N. Antimicrobial Activity of Black Pepper (Piper nigrum L.). Glob J Pharmacol, 2013; 7(1): 87–90.
32. Maitra J, Shilpi. Synergistic Effect of Piperine, Extracted from Piper nigrum, with Ciprofloxacin on Escherichia coli , Bacillus subtilis. Der Pharm Sin, 2017; 8(3): 29–34.
33. Agbor GA, Vinson JA, Oben JE, Ngogang JY. Comparative analysis of the in vitro antioxidant activity of white and black pepper. Nutr Res., 2006; 26(12): 659–63.
34. Sepehri Z, Bagheri G, Javadian F, Anbari M, Nasiri AA, Kiani Z, et al. Antibacterial Activity of Cuminum cyminum and Piper nigrum against antibiotic resistant Klebsiella pneumonia. Antibact Act Cuminum cyminum Piper nigrum against Antibiot Resist Kleb pneumonia, 2014; 3(V): 17–9.
35. Khan M, Siddiqui M. Antimicrobial activity of piper fruits. Indian J Nat Prod Resour, 2007; 6(2): 111–3.
36. Pundir RK, Jain P. Comparative Studies on the Antimicrobial Activity of Black Pepper (Piper Nigrum) and Turmeric (Curcuma Longa) Extracts. Int J Appl Biol Pharm Technol, 2010; 1(2): 492–501.
37. Khalaf NA, Shakya AK, Al-Othman A, El-Agbar Z, Farah H. Antioxidant activity of some common plants. Turkish J Biol, 2008; 32(1): 51–5.
38. Jyothiprabha V, Venkatachalam P. Preliminary Phytochemical Screening of Different Solvent Extracts of Selected lndian Spices. Int J Curr Microbiol Appl Sci., 2016; 5(2): 116–22.
39. Mariselvi S, Manimegalai K. Phytochemical Screening And Xrd Analysis Of Black Pepper Piper Nigrum. [cited 2019 March 12]; Available from: http://dx.doi.org/10.24327/ijcar.2017.4994.0629.
40. NN A. A Review on the Extraction Methods Use in Medicinal Plants, Principle, Strength and Limitation. Med Aromat Plants [Internet]. 2015 Jul 6 [cited 2019 March 12]; 04(03): 1–6.
41. Coe FG, Anderson GJ. Screening of medicinal plants used by the Garífuna of eastern Nicaragua for bioactive compounds. J Ethnopharmacol, 1996;
42. Test for Reducing Sugars | Sciencing [Internet]. [cited, 2019. March 14]. Available from: https://sciencing.com/test-reducing-sugars-5529759.html.
43. Biochemistry Post Lab Finals [Internet]. [cited, 2019. May 13]. Available from: https://www.slideshare.net/Farlogy/biochemistry-post-lab-finals
44. Robiel Ermias, Merih Tukue AB. Journal of Atoms and Molecules. J Atoms Mol, 2011; 1(1): 41–7.
45. Tannins - an overview | ScienceDirect Topics [Internet]. [cited 2019 May 13]. Available from: https://www.sciencedirect.com/topics/biochemistry-genetics-and- molecular-biology/tannins.
46. Tests for Flavonoids | Sodium Hydroxide | Alkene [Internet]. [cited 2019 May 15]. Available from: https://www.scribd.com/document/355656848/Tests-for-Flavonoids.
47. Gupta A, Gupta M, Gupta S. Isolation of piperine and few sesquiterpenes from the cold petroleum ether extract of Piper nigrum (Black pepper) and its antibacterial activity. Int J Pharmacogn Phytochem Res., 2013;
48. Penecilla GL, Magno CP. Antibacterial activity of extracts of twelve common medicinal plants from the Philippines. J Med Plants Res., 2011;
49. F Nikhat, Satyanarayana D, Evs S. Isolation, Charectrisation and Screening of Antioxidant Activity of the Roots of Syzygiumcuminii (L) Skeel. Asian J Res Chem, 2009;
50. Kapoor IPS, Singh B, Singh G, De Heluani CS, De Lampasona MP, Catalan CAN. Chemistry and in vitro antioxidant activity of volatile oil and oleoresins of black pepper (Piper nigrum). J Agric Food Chem, 2009; 57(12): 5358–64.
51. Kähkönen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, et al. Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem, 1999.
52. Gülçin I, Beydemir Ş, Alici HA, Elmastaş M, Büyükokuroğlu ME. In vitro antioxidant properties of morphine. Pharmacol Res., 2004.