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Investigation of Inhibitory Activity and Bioactive Compounds of *Piper nigrum* Seeds on Selected Human Pathogens

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ABSTRACT

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With the increasing incidents of new and reemerging infectious diseases, discovery and urgent need of new microbial compounds having diverse chemical structures and novel mechanism have also been increased. The study focused on determining the antibacterial properties of *P. nigrum* against pathogenic bacteria and analyzing the phytochemical constituents responsible for these medicinal activities. The plant seeds were collected, shade dried and powdered and subjected to extraction by Cold maceration in various solvents. The extracts were used to determine the antibacterial activity by agar well diffusion method and MIC assay. The phytochemistry was also analyzed. Data obtained showed that the ethyl acetate extract was found to be most effective against the selected bacterial cultures. Phenolic content was found as a major phytochemical constituent. On the basis of the Data obtained it is clear that plant extracts can be used as effective herbal cure against human pathogens.

Introduction

Piper nigrum is well known for its bactericidal, cytotoxic and pharmacological benefits. Medicinal plants are various plants thought by some to have medicinal properties, but few plants or their phytochemical constituents have been proven by rigorous science to have medicinal effects. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties (Ganesh *et al.*, 2014). *Piper nigrum* is originally a native to Southwestern India, also known as Malabar Coast. This region is comprised of lush tropical evergreen forests that are very humid and mountainous. Black pepper is now also

cultivated in Cochin-China, East and West Indies, Malay Peninsula, Malay Archipelago, Siam, Malabar and other tropical areas around the world. Plant is perennial, woody climber having height up to 10m or more. Leaves are alternately arranged and almond shaped with tapered tip. They are dark green and shiny above but paler green below. Length of leaf petiole is higher in runner shoots as compared to lateral. Varieties having lower leaf petiole length at the top and increased petiole length at the bottom, both in runner as well as in lateral branches are considered to be ideal for harvesting maximum light. A black pepper plant mostly has monoecious and bisexual

flowers. Cluster of flowers grow on a flowering stalk called spikes. Flowers are whitish to yellow-green in color. About 50-150 flowers are present on a single spike. The fruit of black pepper is spicy and aromatic. They are round and berry-like.

Medicinal plants have been used for years for the preparation of traditional medicines from their natural products which possess antimicrobial activities and thus can cure different diseases (Sharma *et al.*, 2013 and Yang *et al.*, 2009). Plants having medicinal and spice properties are renewable raw material and are of great economic importance. Dried substances of plants or vegetables, which can be whole, broken or in ground form, having fragrant, aromatic or pungent properties can be defined as Spices. Spices contribute in seasoning and flavouring food and beverages rather than providing nutrition (Draughon, 2004). Plant parts that serves as spices includes leaves (coriander, mint), flower (clove), bulbs (garlic, onion), stem (cinnamon), rhizomes (ginger, turmeric) and fruits (red chili, black pepper) (Cown, 1999). It was estimated that majority of the world population (about 80%), to meet their demand, depends on medicines that are derived from plant products (Haq, 2004). One of the main reason of the wide use of plant based products or plant extracts as food preservatives against food borne pathogens is the presence of antibacterial and antifungal activities. These activities are due to the presence of some compounds like isoflavones, anthocyanins and flavonoids (Cown, 1999).

Pepper is native to India and since ancient times has been a prized spice. Due to its antimicrobial property, pepper had been used for the treatment of several diseases like congestion, fever, paralytic, arthritic disorder, vertigo, chronic indigestion, colon toxins, obesity, sinusitis and even diarrhoea and

cholera (Saranraj *et al.*, 2014). In plants, the capability of production of definite physiological action on disease causing organisms is due to the presence of secondary metabolic compounds such as flavonoids, steroids, resins, alkaloids, steroids, tannins, phenol compounds and fatty acids.

For the discovery of new drugs that are effective in treating several diseases, the screening of plant extracts has been of great interest to scientist (Mahesh *et al.*, 2008). Antibacterial effects of *P. nigrum*, ethyl acetate, methanolic and aqueous extracts have been evaluated against some Gram positive and Gram negative bacteria responsible for causing food poisoning and food borne infections by Costa *et al.*, 2010 and Lund *et al.*, 2011 and selected microbes were *Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*. Phytotherapeutics as an alternative tool can be used as microbial agents to control microbial infections in the resistant cases as they are cost-effective and environmentally safe (Schelz *et al.*, 2010).

Thus, for the development of novel chemotherapeutic agents, medicinal plants can be utilized as a potential reservoir (Yang *et al.*, 2009). In our present study, medicinal properties of *P. nigrum* seed extracts were exploited against various infections caused by Gram positive and gram negative bacteria and phytochemical analysis was also done to find out the active ingredients responsible for the antibacterial activity.

Materials and Methods

Plant material and extraction

The dried mature seeds of *Piper nigrum* purchased from local market of Dehradun, India. Twenty five grams of seeds powder

was taken and soaked in 250ml of organic solvents (Ethyl acetate, Methanol) and water for extraction using Cold Maceration method (Akhtar *et al.*, 2014). All the extracts were made solvent free and concentrated using rotary evaporator and preserved at 4°C in airtight bottle until further use.

Chemicals and Reagents

The chemicals and reagents used for the study are of pure grade. Ethyl acetate, Methanol, 20% Acetic acid, Conc. Ammonium hydroxide, Folin- Ciocalteace reagent, Dil. Folin-phenol reagent (1:1 ratio with water), Sodium carbonate (20%), Gallic acid, Sodium nitrate (5%), Aluminum chloride (10%), Sodium hydroxide (4%), Polyvinyl pyrrolidone, Vanillin reagent (800gm of vanillin in 10ml of 99.5% ethanol), Sulphuric acid (72%), Metaphosphoric acid (1%), 2,6-dichloroindophenol sodium salt hydrate, L-Ascorbic acid and Distilled water.

Microorganisms

Five different microorganisms representing Gram-positive and Gram-negative bacteria were used in this study. The two gram-positive bacteria were- *Bacillus subtilis* and *Staphylococcus aureus* while the three gram-negative bacteria were-*Escherichia coli*, *Pseudomonas* and *Salmonella typhi*.

Antimicrobial activity

Antibacterial activities of all the extracts (Ethyl acetate, Methanol, Aqueous) of *P. nigrum* were determined by agar well diffusion method (Chauhan, Neha. *et al.*, 2012). The extracts were dissolved in DMSO (dimethylsulphoxide) to obtain 0.5mg/100µl and 1mg/100µl concentration. Commercial antibiotic (Gentamicin) and DMSO was used as positive and negative control respectively. The test was performed in triplicates and the

final results were presented as the mean zone of inhibition.

Broth dilution MIC tests (NCCLS, 1993)

This test was done to check the zone of inhibition which was minimum. This process is named as macro broth dilution assay. Muller- Hinton broth diluents was taken and 2-fold serial dilutions of all extracts were prepared in the well on the basis of result obtained from agar well diffusion method.

Gentamicin and DMSO are positive and negative control respectively 20µl of test culture at concentration (5×10^5 cfu/ml) was inoculated and plates were incubated for 24h at 37°C.

Plate with minimum growth was taken and concentration is noted as minimum inhibitory concentration. Another value minimum bacterial count was calculated by spreading 20µl of MIC test broth on a new plate incubating for 18- 24h at 37°C. Dilution of plates showing no single bacterial growth was taken as MBC concentration. Triplicates were used to perform. Test and mean MIC and MBC value were calculated and noted.

Phytochemical analysis

Qualitative analysis

Qualitative analysis was done in accordance to (Sharma *et al.*, 2014).

Test for glycosides (Sulphuric acid test)

To 1ml of plant extract, few drops of sulphuric acids were added and the mixture was allowed to stand for few minutes. Presence of glycosides was confirmed by the formation of Reddish precipitate.

Test for carbohydrates (Molisch's test)

To 1ml of extract, 2ml of Molisch's Reagent was added. Now to this mixture, 2ml conc. sulphuric acid was added along the sides of the test tube. Presence of carbohydrates was confirmed by the formation of reddish violet ring at the junction of two liquids.

Test for flavonoids (Aqueous test)

To 1ml of plant extract, add 1ml of aqueous NaOH. Yellow colour formation showed the presence of flavonoids.

Test for saponins (Aqueous test)

To 1ml of extract add 5ml water and shake well in a test tube shaker. Lather formation shows the presence of saponins.

Test for tannins (Ferric chloride test)

To 1ml of plant extract add 1ml of ferric chloride. Formation of greenish black colour confirmed the presence of tannins.

Test for alkaloids (Dragendroff's reagent)

To 1ml of plant extract add 5-6 drops of Dragendroff's reagent. Formation of creamish/ brownish-red/ orange precipitate confirmed the presence of alkaloids.

Quantitative analysis

Quantitative tests were used to detect the presence of phenolics, saponins, flavonoids and ascorbic acid (Sharma *et al.*, 2014)

Test for total phenolics content

The total phenolics content of *P. nigrum* was estimated using Folin- Ciocaltece reagent by

the method of Sidduraju and Becker. About 20 microgram of leaf and root extracts was taken separately and it was made up to 1ml with distilled water. Then 500 microliter of diluted Folin-phenol reagent (1:1 ratio with water) and 2.5ml of sodium carbonate Na₂CO₃ (20%) were added. The mixture was shaken well and incubated in dark condition for 40min. for the development of colour. After incubation, the absorbance was measured at 725nm. A calibration curve of Gallic acid was constructed and linearity was obtained in the range of 10-50microgram/ml. The total phenolics content in the plant extracts were expressed as mg of Gallic acid equivalent (mg GAE/g extract) by using the standard curve.

Test for Flavonoids content

The total flavonoids content was estimated using the procedure described by Zhishen *et al.*, A total of 1ml of plant extracts were diluted with 200 microliter of distilled water separately followed by the addition of 150 microliter of sodium nitrite (5%) solution. This mixture was incubated for 5min and then 150 microliter of aluminium chloride (10%) solution was added and allowed to stand for 6min. Then 2ml of sodium hydroxide (4%) solution was added and made up to 5ml with distilled water. The mixture was shaken well and left it for 15min at room temperature. The absorbance was measured at 510nm. Appearance of pink colour showed the presence of flavonoids content. The total flavonoids content was expressed as rutin equivalent mg RE/g extract on a dry weight basis using the standard curve.

Test for total saponins content

Estimation of total saponins content was determined by the method described by Makkaret. All based on Vallin-sulphuric acid colorimetric reaction with some modifications. About 50 microliter of plant

extract was added with 250 microliter of Vanillin reagent (800 mg of vanillin in 10ml of 99.5% ethanol) was added. Then 2.5ml of 72% sulphuric acid was added and it was mixed well. This solution was kept in a water bath at 60C for 10min. After 10min, it was cooled in ice cold water and the absorbance was read at 544nm. The values were expressed as diosgenin equivalent (mg DE/g extract) derived from a standard curve.

Results and Discussion

Antimicrobial activity

The antibacterial activity of the plants was evaluated using agar well diffusion method. All the seed extracts of *P. nigrum* showed the antibacterial activity against the selected microbes. Gentamicin used as a positive control showed the maximum ZOI against B1 for both the concentrations of 0.5mg/100µl and 1mg/100µl *i.e.* 31mm and 35mm respectively followed by B3, B2, B4 and B5. For 0.5mg/100µl concentration of E1 extract, B1 and B4 showed the maximum ZOI *i.e.* 21mm followed by B2 (ZOI-17mm), B5 (ZOI-16mm) and the minimum zone of inhibition was of B3 *i.e.* 14mm. For 1mg/100µl concentration of E1 extract the most susceptible bacteria was B1 with ZOI of 24mm. It was followed by B4 (ZOI-21mm), B2 (ZOI-19mm), B5 (ZOI-18mm) and the B3 which was the least susceptible bacteria having zone of inhibition 16mm. For 0.5/100µl mg concentration of E2 extract, B4 and B5 showed the maximum ZOI *i.e.* 18mm and 14mm respectively. B3 have the ZOI of 10mm and the B1 and B2 have the same ZOI *i.e.* 8mm which makes them least susceptible. For 1mg/100µl concentration of E2 extract, B1, B2 and B3 showed least susceptibility having zone of inhibition of 9mm, 9mm and 12mm respectively. The highest susceptibility was observed in B4 having zone of inhibition of 22mm followed by B5 having zone of inhibition of 16mm. This shows that the B4 is

the most susceptible while the B2 and B1 are the least susceptible for E2 extract. For 0.5mg/100µl concentration of E3 extract, B1 and B5 have maximum zone of inhibition *i.e.* 8mm followed by B2 and B4 which have zone of inhibition of 7mm and B3 have the least zone of inhibition *i.e.* 6mm. For 1mg/100µl concentration, the maximum zone of inhibition was observed for B1 *i.e.* 10mm followed by B4 having zone of inhibition of 9mm. The zone of inhibition for B2, B3 and B5 is equal *i.e.* 8mm. So it shows that the B1 is the most susceptible bacteria for the E3 extract of seeds of *P. nigrum*. The results obtained were tabulated in tables 1, 2, 3, 4 and 5. The extracts showing high efficacy against selected pathogens were subjected to minimum inhibitory concentration (MIC) assay by two-fold serial dilution method (2:2)

Qualitative analysis

The various plant extracts of *P. nigrum* were phytochemically assessed using qualitative as well as quantitative methods. Phytochemical analysis of *P. nigrum* ethanolic extract showed the presence of Glycosides, carbohydrates, flavonoids, saponins, tannins and alkaloids. Methanolic extract of *P. nigrum* also showed the presence of glycosides, carbohydrates, flavonoids, saponins, tannins and alkaloids. Aqueous extract of *P. nigrum* showed the presence of glycosides, carbohydrates, flavonoids, saponins, tannins and alkaloids. Results are tabulated in table 5.

Quantitative analysis

The phytochemical assessment of various plant extracts was done using quantitative methods. The phenolic content was found to be highest in E1 extract *i.e.* 0.591 followed by E3 *i.e.* 0.556 and then the E2 extract *i.e.* 0.515. The content of saponins was highest in

E3 extract *i.e.* 0.044. E2 extract showed the highest flavonoid content *i.e.* 0.015. The saponin content was found to be highest in E3 (0.044) extract of *P. nigrum* followed by E2 (0.035) and then E1 (0.025). The flavonoid content was present highest in E2 extract (0.015) followed by E1 (0.009) and E3 (0.007). The results of quantitative screening of different extracts have been tabulated in table 6 and figure 2.

MIC

MIC is the lowest concentration of the test sample or drug at which it shows the highest inhibitory activity against microorganisms. The extracts showing high efficacy against

microorganisms were subjected to minimum inhibitory concentration (MIC) assay by two-fold serial dilution method (2:2) (Florey *et al.*, 1989 and Drummond *et al.*, 2000). From the data obtained it was observed that the MIC value ranged from 0.0156-0.625mg/ml. To determine whether an extract is bactericidal (MIC/MBC <4) or bacteriostatic (MIC/MBC >4) in nature MIC Index (MIC/MBC) was performed. The E1 extract showed the lowest concentration of MIC for B2 *i.e.* 0.625mg/ml. MIC concentration for E2 extract was found to be lowest for B3 *i.e.* 0.125mg/ml and the E3 extract showed the same MIC value for all the selected microbes *i.e.* 0.25mg/ml (Fig. 1)

Fig.1 Represents antibacterial activity of *P. nigrum* E1 extract against various bacterial cultures

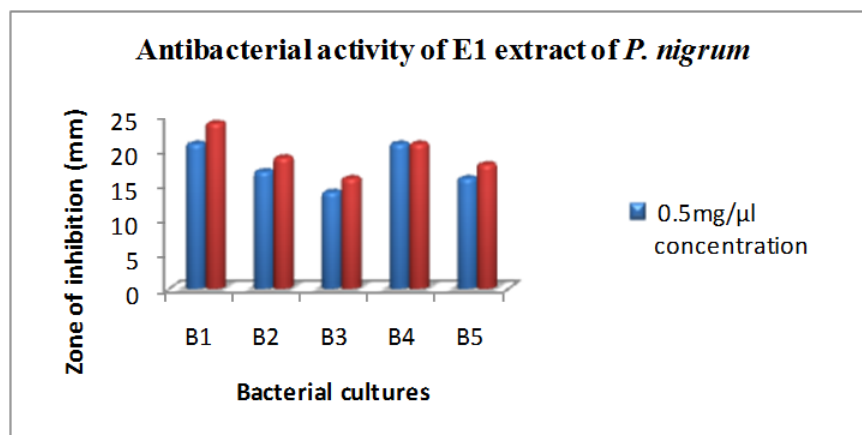


Fig.2 Represents the concentration phytochemicals in various extracts of *P. nigrum*

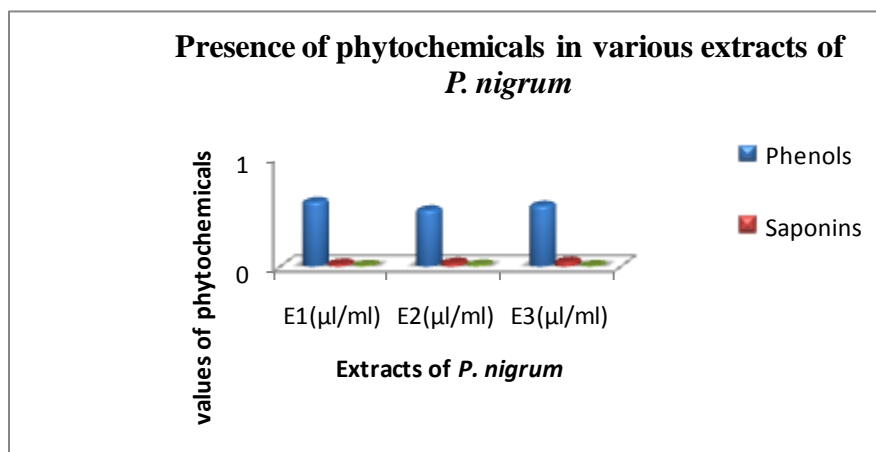


Table.1 The yield and physical properties of *Piper nigrum* extracts

S. No.	Solvent Used	Yield (g/250ml)	Colour	State
1.	Ethyl Acetate (E1)	3.41	Blackish brown	Viscous
2.	Methanol (E2)	1.64	Blackish brown	Viscous
3.	Water (E3)	2.12	Brownish	Solid

Table.2 Antibacterial activity of Gentamicin

S. No.	Bacterial cultures	ZOI (0.5mg Concentration)	ZOI (1mg Concentration)
1.	B1	31mm	35mm
2.	B2	26mm	30mm
3.	B3	27mm	32mm
4.	B4	24mm	28mm
5.	B5	22mm	27mm

Table.3 Antibacterial activity of *P. nigrum* E1 extract

S. No.	Bacterial cultures	ZOI (0.5mg Concentration)	ZOI (1mg Concentration)
1.	B1	21mm	24mm
2.	B2	17mm	19mm
3.	B3	14mm	16mm
4.	B4	21mm	21mm
5.	B5	16mm	18mm

Table.4 Antibacterial activity of *P. nigrum* E2 extract

S. No.	Bacterial cultures	ZOI (0.5mg Concentration)	ZOI (1mg Concentration)
1.	B1	8mm	10mm
2.	B2	8mm	10mm
3.	B3	10mm	12mm
4.	B4	18mm	22mm
5.	B5	14mm	16mm

Table.5 Antibacterial activity of *P. nigrum* E3 extract

S. No.	Bacterial cultures	ZOI (0.5mg Concentration)	ZOI (1mg Concentration)
1.	B1	8mm	10mm
2.	B2	7mm	8mm
3.	B3	6mm	8mm
4.	B4	7mm	9mm
5.	B5	8mm	10mm

Table.6 The MIC, MBC and MIC Index values of E1 extract against different pathogens

Organisms	Range (mg/ml)	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
B1	0.5-0.0156	0.0156	0.0312	0.125	0.25	2	2
B2	0.5-0.0156	0.0156	0.0312	0.625	0.125	2	0.2
B3	0.5-0.0156	0.0156	0.0312	0.125	0.25	2	2
B4	0.5-0.0156	0.0156	0.0312	0.125	0.25	2	2
B5	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2

Table.7 The MIC, MBC and MIC Index values of E2 extract against different pathogens

Organisms	Range (mg/ml)	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
B1	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2
B2	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2
B3	0.5-0.0156	0.0156	0.0312	0.125	0.25	2	2
B4	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2
B5	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2

Table.8 The MIC, MBC and MIC Index values of E3 extract against different pathogens

Organisms	Range (mg/ml)	MIC (control) (mg/ml)	MBC (control) (mg/ml)	MIC (extract) (mg/ml)	MBC (extract) (mg/ml)	MIC Index (control)	MIC Index (extract)
B1	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2
B2	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2
B3	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2
B4	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2
B5	0.5-0.0156	0.0156	0.0312	0.25	0.5	2	2

Table.9 Represents the presence of different phytochemicals in various extracts of *P. nigrum*

S. No.	Phytochemicals	<i>Piper nigrum</i>		
		E1 extract	E2 extract	E3 extract
1	Glycosides	Present	Present	Present
2	Carbohydrates	Present	Present	Present
3	Flavonoids	Present	Present	Present
4	Saponins	Present	Present	Present
5	Tannins	Present	Present	Present
6	Alkaloids	Present	Present	Present

Table.10 Quantitative analysis of *P. nigrum* various extracts

Extracts	Phytochemicals		
	Phenol	Saponins	Flavonoids
E1 (µg/ml)	0.591	0.025	0.009
E2 (µg/ml)	0.515	0.035	0.015
E3 (µg/ml)	0.556	0.044	0.007

Antibacterial activity

Extracts of the seeds of *P. nigrum* were subjected for antibacterial study. B3 was found to be least susceptible as it showed the minimum zones of inhibition against all the three extracts (E1, E2 and E3) of *P. nigrum*. E1 extract was found to possess strong inhibitory activity against B1 followed by B4, but moderately inhibit the growth of B2 and B5 and least effective against B3. E2 extract showed maximum ZOI against B4 followed by B5, B3, B1 and B2. E3 extract effectively inhibit the growth of B1 and B5 in comparison with B4, B2 and B3. In the other studies done on *P. nigrum*, methanolic extract was found have strong antibacterial activity against *E. coli* but our study showed that E1 extract of *P. nigrum* possess strong inhibitory activity against all the selected bacteria as compared to other two extracts. Abdel Gadir (2007) reported that water extract of *P. nigrum* have no activity against *E. coli* and *Pseudomonas* but results of our study have shown good activity of E3 extract against both the bacteria.

MIC

Different extracts of *P. nigrum* showed varied MIC values. E1 extract was found to have strong inhibitory concentration as compared to other extracts. B2 was found to be strongly inhibited by E1 extract. In E2 extract, B3 showed lowest MIC values while in E3 extract all the bacteria possess equal MIC values. In the study done by Akhtar *et al.*, 2014, ethanol and methanol leaf extract of *P. nigrum* was found to have MIC concentration of 12.5mg/ml. The extract was considered as bacteriostatic if the MIC Index values are greater than 4 and less than 32. On the basis of the above data, all the extracts were found to have bactericidal properties.

Phytochemical screening

The E1, E2 and E3 extracts obtained from the seeds of *P. nigrum* were found to be strongly active against the selected human microbes.

Phytoconstituents have four type of bactericidal effects: 1. they inhibit cell wall synthesis 2. They stop microbial protein and nucleic acid synthesis 3. They disrupt microbial membrane structure and function 4. They block metabolic pathways through inhibition of key enzymes (Latha *et al.*, 2006 and Zulfiler *et al.*, 2011). The various extracts of *P. nigrum* have exhibited strong inhibitory activity against the selected microbes. These microbes cause various types of food borne infections and food poisoning. Piperine, which is an alkaloid present in seeds of *P. nigrum*, was reported as a major phytochemical in the study done by Hamrapurkar *et al.*, 2011. But in our study phenolic content was found as a major phytochemical in all the three extracts of *P. nigrum* followed by Saponins and Flavonoids. All these phytochemicals were also reported by Kumar *et al.*, 2014 in the phytochemical screening of ethanolic extract of *P. nigrum* seeds (Tables 7–10).

In conclusion, Medicinal plants possess many phytochemicals and serve as phytotherapeutic agents for curing various diseases. They have no or negligible effects on the human body as compared to antibiotics which can provide immediate relief but also have numerous side effects. The findings of our result showed that the various extracts of *P. nigrum* have showed effectiveness as antibacterial effects. As compared to other studies, our data showed that the Ethyl acetate extract of *P. nigrum* possess strong antimicrobial activity against selected gram positive and gram negative microbes. The identified phytochemicals can be used for the formulation of herbal drugs to cure various food borne diseases as they are cost effective, environment friendly and have negligible side effects.

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