



Evaluation of Antibacterial Activity of *Piper betel* Leaves and *Nigella sativa* Seeds against Multidrug Resistant Food and Water Borne Pathogenic Bacteria: An *in vitro* Study Model

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MSR, TI and MSH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MAW and EMSB managed the analyses of the study. Author AMT managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This research was aimed to contribute to the previous knowledge of combating pathogens by using local medicinal plants in Bangladesh. In the present work, the antibacterial activity of Paan (*Piper betel* leaves) and Black cumin (*Nigella sativa* seeds) extracts in water as well as in various organic solvents (namely methanol and ethanol) were analyzed against food and waterborne pathogenic bacteria (both gram-positive and gram-negative) viz. *Staphylococcus aureus*, *Bacillus cereus*,

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Escherichia coli, *Salmonella typhi*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, all of which were previously found to be resistant to different antibiotics. Methanol extracts of *Piper betel* was the best extract in inhibiting the growth of gram-positive bacteria, secondly aqueous and ethanol extracts were less effective as antibacterial agents except against *S. aureus* while gram-negative bacteria, only *S. typhi* and *P. aeruginosa* were found to be sensitive to aqueous extracts. Methanol extracts of Black cumin was effective against gram-negative where ethanol extracts were effective against gram-positive but *S. typhi* was sensitive to both ones. When extracts were compared with the traditional antibiotics, this activity was especially competent against *S. aureus* followed by *B. cereus* and *B. subtilis*. When Betel extracts were compared with the traditional antibiotics, this activity was especially competent against *E.coli* followed by *S. typhi* and *P. aeruginosa*. The broth dilution assay revealed that the bactericidal values for both the plant extracts fall in the range of 1125 to 2250 µg/ml. These results suggest that betel leaves and black cumin seeds have potential antibacterial activity against multiple antibiotic-resistant bacteria.

Keywords: *Piper betel*; *Nigella sativa*; antibacterial activity.

1. INTRODUCTION

In developing countries food poisoning is considered as one of the most common causes of illness and death [1,2]. Gram negative bacteria like *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa* are associated with most of food poisoning reports [3,4]. On the Other hand, Gram positive bacteria mainly include *Staphylococcus aureus* and *Bacillus cereus* that have been also identified as the causal agents of food borne diseases or food spoilage [5]. Chemical preservatives have been used traditionally to prevent food spoilage and their etiological agent [6,7]. Though these chemical preservatives are efficient enough in prevention and outbreak control of food poisoning diseases, repeated applications of these hazardous chemicals have resulted in the accumulation of chemical residues in food and feed chains. This situation resulted on acquisition of microbial resistance to these applied chemicals and unpleasant side effects of these chemicals on human health [8,9].

The emergence and spread of multidrug resistant (MDR) bacterial pathogens is also a similar situation. These MDR bacterial pathogens have substantially threatened the existing antibacterial therapy [10]. Despite of the large production of a number of new antibiotics by the pharmacological industries, resistance to these drugs by microorganisms has increased in the last three decades. In general, bacteria have the genetic capability to transmit and acquire resistance to drugs by genetic modification of their DNA which are utilized as therapeutic agents [11,12]. Plants have been valuable and indispensable sources of natural products for the health of human beings having a great potential

for producing new drugs from a long periods of time [13–15]. It has been estimated that between 60-90% of the populations of developing countries use traditional and botanical medicines exclusively and consider them to be a normal part of primary healthcare [12]. As medicinal plants contain components of therapeutic value, they have been used for centuries as remedies for human ailments and diseases [16]. As they produce less pollutants produced in production and have minimal health hazards, so they are less toxic to humans and environmentally friendly [17]. The *P. betel* Linn. (Betel leaf), a vine belonging to the Piperaceae family is commonly known as paan in Bangladesh and largely distributed in tropical and subtropical regions of the world [18]. Activities possessed by the leaves of *P. betel* include antidiabetic, antiulcer, antiplatelet aggregation, antifertility, cardiogenic, antitumour, antimutagenic, respiratory depressant [19,20,21] and also used as carminative, stomachic, anthelmintic, tonic, and aphrodisiac [18]. Many reports have disclosed that the leaf of this plant contains many beneficial bioactivities and its extract has a great potential to be used in the development of commercial products [22]. *Nigella sativa* is a herbal plant. It is better known as black seed or locally known as kaligira in Bangladesh. It is found in Southeast Asia and Mediterranean countries. In Indian subcontinent this plant is used as a food preservative as well as a protective and curative treatment for numerous disorders [22]. The black seeds of *Nigella sativa* contain 36–38% fixed oil as well as proteins, alkaloids, saponins and essential oils making up the rest of the composition [23]. Moreover black seed extract or oil has been reported to possess antimicrobial activity [24], antioxidant activity [25], antitumor activity [26] and a stimulatory effect on the

immune system as well as potential as an antimicrobial agent has not been exploited. The production of new and potent antibacterial agent is needed urgently, mainly for hospitals and health facilities as pathogenic multidrug resistant bacteria are emerging day by day. Thus, the evaluating study aims to assess the antibacterial activity of *P. betel* leaves and *Nigella sativa* seeds in an *in vitro* study model which may be helpful in developing new novel drugs.

2. MATERIALS AND METHODS

2.1 Plant Selection and Preparation

The selection of two medicinal plants such as Paan (*Piper betle*), Kalogira (*Nigella sativa*) was used based on their reported traditional use. All the plants were easily accessible and readily available in the market. *Piper betle* leaves and *Nigella sativa* seeds were purchased from the local market from Ullapara in Sirajgonj. Fresh healthy leaves and seeds washed and air dried. Dried leaves shredded into small pieces & both samples were grounded. The powdered betel leaves and kalogira seeds were kept in sterile plastic bags until further used. The plants were identified in the national Herbarium from stored sample.

2.2 Microorganisms

Six different bacteria were used obtained from the stock culture of the Department of Microbiology, Primeasia University such as *E. coli*, *S. aureus*, *S. typhi*, *P. aurigenosa*, *B. cereus*, and *B. subtilis*.

2.3 Preparation and Maintenance of Stock Cultures

The isolated bacteria were inoculated on nutrient agar slopes and incubated overnight at 37°C. This strain were grown in nutrient agar at 37°C and agar slant, then again grow in nutrient broth for 3-4 hours.

2.4 Plant Extraction

An extraction method adapted from Eloff (2000) [27] was employed using 100 g of freshly processed plant leaves of *Piper betle* and seeds of *Nigella sativa* for the two plants. A schematic representation of the extraction procedure illustrates the different steps performed to obtain

the plant extract supernatants. Three extraction procedure was performed as follows with the fresh plant material.

2.5 Methanol Extract of Kalogira

At first 13.6 g sample were measured and mixed with 54.4 ml methanol, then mixed well and stored at 25°C for overnight. After that the solution was filtered and filtrate were taken and dried at 45°C in a shaker incubator for 2 days. Extract resuspended in methanol, 0.1 g sample was added in 18ml methanol. Final concentration were 18 mg/ml.

2.6 Ethanol Extract of Kalogira

At first 13.6 g sample were measured and mixed with 54.4 ml methanol, then mixed well and stored at 25°C for overnight. After that the solution was filtered and filtrate were taken and dried at 45°C in a shaker incubator for 2 days. Extract resuspended in ethanol, 0.1 g sample was added in 18ml ethanol. Final concentration were 18 mg/ml.

2.7 Methanol Extract of Paan

At first 10 g sample were measured and mixed with 50 ml methanol, then mixed well and stored at 25°C for overnight. After that the solution was filtered and filtrate were taken and dried at 45°C in a shaker incubator for 2 days. Dried extract resuspended in methanol, 0.1 g sample was added in 10 ml methanol. Final concentration were 10 mg/ml.

2.8 Ethanol Extract of Paan

At first 10 g sample were measured and mixed with 50 ml methanol, then mixed well and stored at 25°C for overnight. After that the solution was filtered and filtrate were taken and dried at 45°C in a shaker incubator for 2 days. Dried extract resuspended in ethanol, 0.1 g sample was added in 10 ml ethanol. Final concentration were 10 mg/ml.

2.9 Water (Aqueous) Extract of Paan

At first 10 g sample were measured and mixed with 100 ml sterile water, then mixed well and boiled at 100°C. It was dried at 45°C in a shaker incubator for 2 days dried extract resuspended in sterile water, 0.1 g sample was added in 10 ml sterile water. Final concentration were 10 mg/ml.

2.10 Antibiotic Susceptibility Test

Antibiotic susceptibility of the bacterial isolates were determined by disc diffusion method. A bacterial turbidity equivalent of 0.5 McFarland standards was used as inoculum for each isolate. The antibiotic resistance pattern for the panel of antibiotics were determined considering the zone of inhibition sizes for each of the antibiotics as "resistant (R)" or "sensitive (S)" against the test isolates as recommended by the Clinical and Laboratory Standard institute (CLSI, 2007). The antibiotic discs used here are Vancomycin 30 µg, Amikacin 30 µg, Chloramphenicol 30 µg, Bacitracin 10 µg, Amoxycilin 10 µg, Netilmicin 30 µg.

2.11 Paper Disc Diffusion Method

Bacterial susceptibility to antimicrobial agent was determined in vitro by using the standard agar disc diffusion method known as Kirby Bauer method (Bauer et al. 1966). Culture of six bacteria species such as *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Bacillus subtilis* were prepared by adding an overnight culture of the organism in Muller Hinton (MH) broth to obtain an OD₆₀₀ 0.1. The cells are allowed to grow until they obtain the McFarland standard 0.5 approximately 10⁸ CFU/ml.

Sterile discs (Oxoid) were soaked separately with 18 Mg/ml of organic extract of kalogira prepared in Methanol & Ethanol and 10 Mg/ml of Paan extract prepared in Methanol, Ethanol, Aqueous (Water). It is placed on Muller Hinton (MH) agar plates previously swabbed with target bacteria species at a concentration of 10⁸CFU/ml. Soaked organic solvent and water disc was added as a positive and negative control respectively. Plates were kept at 4°C for 1 1/2 hour for better spreading of the extract materials around the disc and then incubated for 24 hour at 37°C. Antibacterial

activity was defined as the diameter (mm) of the clear inhibitory zone formed around the disc. Kanamycin (30 µl) antibiotic disc was used as a positive control.

2.12 Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Determination

MIC was only determined for ethanolic, methanolic and aqueous extract that showed positive antimicrobial activity. Serial two-fold dilutions were made from the highest concentrations (4.5 mg/ml). Concentration range tested from 4500-17.57 µg/ml. MBC is the lowest concentration from which the microorganisms did not recover. For MBC determination, 100 µl of the three to four last vials showing no turbidity (no growth) from the MIC procedure were sub-cultured onto NA plate. The plates were incubated at 37°C for 24 h. Control samples (positive and negative) were incubated under the same conditions.

3. RESULTS

Fig. 1 shows the antibiotic resistant pattern of the pathogenic bacteria used in this study. The bacterial isolates were collected from the stock culture of Prime Asia University. The antibiotic discs used here are Vancomycin 30 µg, Amikacin 30 µg, Chloramphenicol 30 µg, Bacitracin 10 µg, Amoxycilin 10 µg and Netilmicin 30 µg. Fig. 1 shows that all the test bacteria are resistant to 16.67% to 66.67% antibiotics.

Table 1 shows the antibacterial activities of the crude extracts of betel leaves on pathogen at 10 mg/ml concentration. The crude extracts of betel leaves on gram negative bacteria were less effective compared to the gram positive bacteria. The most sensitivity was found against *S. aureus* followed by *B. subtilis*.

Table 1. Antibacterial Activities of the Crude Extracts of *Piper betle* (Betel Leaves) on Pathogen at 10 mg/ml

Organisms	Water	Methanol	Ethanol	Positive control	Negative control
<i>E. coli</i>	NI	NI	NI	+++	NI
<i>P. aurigenisa</i>	+	NI	NI	+++	NI
<i>S. aureus</i>	+++	++	+++	+++	NI
<i>S. typhi</i>	++	+	++	+++	NI
<i>B. cereus</i>	++	+	+	+++	NI
<i>B. subtilis</i>	++	+++	+	+++	NI

(NI) denote no zone formed, (+) denote small zone formed where zone size is less than 13 mm, (++) denote medium zone formed where zone size is between 14 to 17 mm, (+++) denote big zone formed where zone size is more than 18 mm

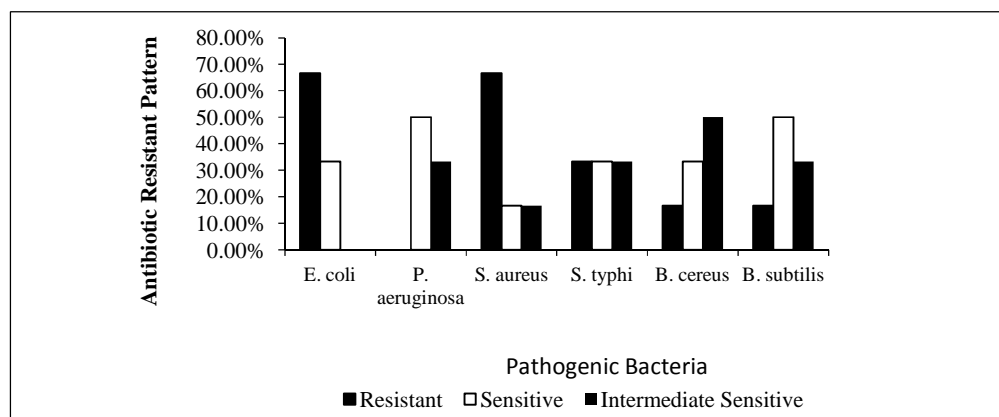


Fig. 1. Antibiotic resistant pattern of the pathogenic bacteria

Table 2 shows the antibacterial activities of the crude extracts of kalogira on pathogen at 18 mg/ml concentration. Between the two gram negative bacteria *E. coli* was more sensitive to methanol extract compared to *P. aeruginosa* but resistant to ethanol extract. Among the gram positive bacteria *S. aureus* was the most sensitive bacteria to methanol extract whereas *S. typhi* showed the most sensitivity against ethanol extract.

Table 3 reveal the MIC & MBC result of *N. sativa* methanol extract against the test bacteria. *S. aureus* showed the lowest MIC value that is 0.5625 mg/ml. Others showed the MIC value of 1.125 mg/ml. The MBC value was 2.25 for all test bacterium.

Table 4 reveal the MIC & MBC result of *N. sativa* ethanol extract against the test bacteria. Just like the methanol extract *S. aureus* showed the lowest MIC value 0.5625 mg/ml. Others showed the MIC and MBC value of 1.125 mg/ml and 2.25 mg/ml respectively.

Table 5 shows the MIC & MBC result of methanol extract of Pan against the test bacteria.

S. aureus showed the lowest MIC value 0.5625 mg/ml. Others showed the MIC value of 1.125 mg/ml. The MBC value was 2.25 for all test bacterium.

Table 6 shows the MIC & MBC result of ethanol extract of Pan against the test bacteria. *S. aureus* showed the lowest MIC value 0.5625 mg/ml. Others showed the MIC value of 1.125 mg/ml. The MBC value was 2.25 for all test bacterium.

Table 7 shows the MIC & MBC result of water extract of Pan against the test bacteria. *S. aureus* showed the lowest MIC value 0.5625 mg/ml. Others showed the MIC value of 1.125 mg/ml. The MBC value was 2.25 for all test bacterium. The result show that MIC was lower in the gram positive bacteria, gram positive bacteria species showed greater sensitivity towards the extracts. The MBC>MIC value of the *P. betle* ethanolic extract against tested microorganisms. MBC values close to MIC values; MBC/MIC ratios of 2 (MBC/MIC <4); indicating microbocidal action. .No colony growth on the solid medium when MBC of plant extract was used against test organism.

Table 2. Antibacterial activities of the crude extracts of *N. sativa* on pathogen at 18 mg/ml

Organisms	Methanol	Ethanol	Positive control	Negative control
<i>E. coli</i>	+++	NI	+++	NI
<i>P. aurigenisa</i>	++	+	+++	NI
<i>S. aureus</i>	++	+	+++	NI
<i>S. typhi</i>	+	+++	+++	NI
<i>B. cereus</i>	NI	++	+++	NI
<i>B. subtilis</i>	+	++	+++	NI

(NI) denote no zone formed, (+) denote small zone formed where zone size is less than 13 mm, (++) denote medium zone formed where zone size is between 14 to 17 mm, (+++) denote big zone formed where zone size is more than 18 mm

Table 3. MIC & MBC result of *N. sativa* methanol extract

Organism	1 (4.5)	2 (2.5)	3 (1.12)	4 (0.56)	5 (0.281)	6 (0.1405)	7 (0.07)	8(0.03)	9 (0.017)
<i>E. coli</i>	-	-MBC	- MIC	+	+	++	++	++	+++
<i>P. aureginosa</i>	-	-MBC	- MIC	+	+	++	++	+++	+++
<i>S. aureus</i>	-	- MBC	-MIC	-	+	+	+	+	+
<i>S. typhi</i>	-	- MBC	-MIC	+	+	++	++	++	+++
<i>B. subtilis</i>	-	- MBC	-MIC	+	+	++	++	+++	+++

(-), refer no growth, (+) denote small slight growth, (++) denote medium growth, (+++) denote heavy growth

Table 4. MIC & MBC result of *N. sativa* ethanol extract

Organism	1 (4.5)	2 (2.25)	3 (1.125)	4 (0.5625)	5 (0.28125)	6 (0.140625)	7 (0.0703)	8 (0.0351)	9 (0.01757)
<i>P. aureginosa</i>	-	- MBC	- MIC	+	++	++	++	++	++
<i>S. aureus</i>	-	- MBC	- MIC	-	+	++	++	+++	+++
<i>B. cereus</i>	-	- MBC	- MIC	+	+	++	++	++	++
<i>B. subtilis</i>	-	- MBC	- MIC	+	++	++	++	++	+++

(-), refer no growth, (+) denote small slight growth, (++) denote medium growth, (+++) denote heavy growth

Table 5. MIC & MBC result of Methanol extract of *P. betle*

Organism	1 (4.5)	2 (2.25)	3 (1.125)	4 (0.5625)	5 (0.28125)	6 (0.140625)	7 (0.0703)	8 (0.0351)	9 (0.01757)
<i>S. typhi</i>	-	- MBC	- MIC	+	+	+	++	++	++
<i>S. aureus</i>	-	- MBC	- MIC	-	+	+	++	++	+++
<i>B. cereus</i>	-	- MBC	- MIC	+	+	++	++	++	++
<i>B. subtilis</i>	-	- MBC	- MIC	+	+	++	++	++	+++

Table 6. MIC & MBC result of ethanol extract of *P. betle*

Organism	1 (4.5)	2 (2.25)	3 (1.125)	4 (0.5625)	5 (0.28125)	6 (0.140625)	7 (0.0703)	8 (0.0351)	9 (0.01757)
<i>S. typhi</i>	-	- MBC	- MIC	+	+	+	+	++	++
<i>S. aureus</i>	-	- MBC	- MIC	-	+	+	+	+	++
<i>B. cereus</i>	-	- MBC	- MIC	+	+	+	++	++	++
<i>B. subtilis</i>	-	- MBC	- MIC	+	+	++	++	+++	+++

(-), refer no growth, (+) denote small slight growth, (++) denote medium growth, (+++) denote heavy growth

Table 7. MIC & MBC result of aqueous (water) extract of *P. betle*

Organism	1 (4.5)	2 (2.25)	3 (1.125)	4 (0.56)	5 (0.28)	6 (0.14)	7 (0.0703)	8 (0.0351)	9 (0.01)
<i>P. aureginosa</i>	-	- MBC	- MIC	+	+	+	++	++	++
<i>S. typhi</i>	-	- MBC	- MIC	+	++	++	++	+++	+++
<i>S. aureus</i>	-	- MBC	- MIC	+	+	++	++	+++	+++
<i>B. cereus</i>	-	- MBC	- MIC	+	+	++	++	+++	+++
<i>B. subtilis</i>	-	- MBC	- MIC	+	++	++	+++	+++	+++

(-), refer no growth, (+) denote small slight growth, (++) denote medium growth, (+++) denote heavy growth

4. DISCUSSION

Since many years, flora have proved to be an important supply of drug and lots of plant life had been screened whether they incorporate compounds with medicinal value or no longer [28]. An alarming increase in bacterial lines proof against a number of antimicrobial agents demands that a renewed effort be made to try to find out antibacterial agents effective towards pathogenic bacteria proof against current antibiotics. Many of those flora were investigated scientifically for antimicrobial activity and a huge range of plant merchandise have been shown to inhibit growth of pathogenic bacteria. A variety of these agents seem to have systems and modes of movement that are distinct from the ones of the antibiotics in contemporary use, suggesting that cross-resistance with agents already in use can be minimum [29]. Therefore, it is very essential to evaluate the antimicrobial activity of *Piper betel* leaves and *Nigella sativa* seeds against multidrug resistant food and water borne pathogenic bacteria. Methanol extracts of *P. betel* was the best extract in inhibiting the growth of gram-positive bacteria, secondly aqueous and ethanol extracts were less effective as antimicrobial agents except against *S. aureus* while in case of gram-negative bacteria, only *S. typhi* and *P. aeruginosa* were found to be sensitive to aqueous extracts. Methanol extracts of Black cumin was effective against gram-negative where ethanol extracts were effective against gram-positive but *S. typhi* was sensitive to both ones. When *P. betel* extracts were compared with the traditional antibiotics, this activity was especially competent against *S. aureus* followed by *B. cereus* and *B. subtilis*. When Betel extracts were compared with the traditional antibiotics, this activity was especially competent against *E. coli* followed by *S. typhi* and *P. aeruginosa*. The broth dilution assay revealed that the bactericidal values for both the plant extracts fall in the range of 1125 to 2250 µg/ml. These results suggest that betel leaves and black cumin seeds have potential antibacterial activity against multiple antibiotic-resistant bacteria. This fact may be explained by the postulate that the crude form of plant extract contains a lower concentration of bioactive compounds [30]. While screening medicinal plants for antimicrobial activity, it is generally expected that a greater number of compounds would be active against Gram positive rather than Gram negative bacteria [31,32]. In a research conducted by Balaji et al. [33] using aqueous and ethanolic extract of leaves of

P. betel indicated that the ethanolic extract of this plant showed better antibacterial activity against *B. subtilis*, *S. aureus* and *E. coli* and moderate activity against *M. luteus* and *P. aeruginosa*. These results are quite similar to that of our present study although the sample preparation and some organisms were different. This may be described by the fact that the secondary metabolites responsible for demonstrating antimicrobial activity are greatly dependent on solvent system and collection process of metabolites from the plant sources [34]. Moreover, the geographical area and environment also affects the chemical composition of the plants and leads to the variation in activity (Girish and Satish, 2008). Again, it was reported by several studies that several phytochemicals like terpenoids, flavonoids, tannins, alkaloids, steroids and some phenolic compounds are responsible for the antibacterial activity of the plant extract [35,36]. A study conducted by Al-Adhroey et al. [37] showed that the methanolic extract of the *P. betel* leaves contains certain phytochemicals like alkaloids, terpenes, anthraquinones, flavonoids, tannins, saponins and steroids. Whatever the mechanism, it is clear that some of these phyto-constituents of the plant extract may be responsible for the antimicrobial activity.

Again, Antimicrobial interest of different extracts can also be attributed to the presence of soluble phenolic and polyphenolic compounds [38]. The outcomes also are in confirmation with a latest observe [39] wherein it became proven that the methanol extract of *Nepetacataria* inhibited the increase of all of the bacterial and fungal check organisms, with most inhibitory outcomes on *S. Aureus*, *P. Multocida* and *E. Coli* and a minimum effect on *A. Flavus*. Thus suggesting that the antimicrobial interest of the extract may be associated with the monoterpenoid aspect i.e., nepeta-lactone. The lack of antibacterial interest in a number of the concentrations of the extract is no longer sudden as a number of plant extracts were determined ineffective towards sure test organisms at decrease concentrations and can be attributed to the presence of lesser amounts of the antimicrobial compounds. This confirms earlier studies which reported antibacterial activity of alcoholic extract of seeds [40], volatile oil obtained by steam distillation of fixed oil, essential oil, methanolic extract of *N. sativa* cell culture, methanolic extract of germinating seeds [41,42] and essential oil (obtained from crude extract). In a study by Sokmen et al. [43] methanolic extract derived by

soxhlet extraction was not active against one strain of *S. aureus* derived from a clinical sample. This may be due to difference of strains tested.

5. CONCLUSION

The research of new ways to control multidrug resistant bacteria is essential to human and animal health because in the last few decades there are evidences of the emergence of multidrug resistant bacteria. *Piper betle* leaf extracts and Black cumine extracts may be part of this situation as these two have significant antimicrobial activity against broad spectrum of microorganisms (Gram-positive and Gram-negative bacteria). Research result of two herbal plant extract show that it is more effective than some commercial drug (Antibiotic). So it seems that if drug is produced using these plant extracts it may be more effective than some commercial drugs. As *Piper betle* leaf and Black cumine are locally available and can be easily cultivated, they may be a good choice for the development of new strategies for therapy of bacterial diseases. However, more research is needed to identify the active compounds, determine toxicity to humans, and develop formulations.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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