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Full Length Research Paper

Antibacterial activity of chloroform and ethanol extracts of black cumin seeds (*Nigella sativa*) against multi-drug resistant human pathogens under laboratory conditions

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Chloroform and ethanol extracts of black cumin seeds (*Nigella sativa*) were analyzed for antibacterial activity against five food and water borne pathogens, namely, *Staphylococcus aureus*, *Bacillus cereus*, *Vibrio cholerae*, *Klebsiella pneumoniae, Escherichia coli,* and one food spoilage bacteria *Bacillus subtilis*, all of which were previously found to be resistant to different antibiotics. The antibacterial activities of the extracts were determined by disc diffusion and tube dilution methods. All the bacterial strains except *E. coli and K. pneumoniae*, showed sensitivity to the chloroform extract as well as the bacterial strains except the *K. pneumoniae* showed sensitivities to ethanol extract. The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of both extracts were also evaluated. Ethanol extract of black cumin was as found to be highly effective for *B. subtilis* (MIC value 375 µg/ml), followed by *S. aureus* (MIC value 1125 µg/ml). The highest concentration of extract was required for *E. coli* for complete inhibition of their growth. MIC value for *E. coli* was 3000 µg/ml. These results suggest that black cumin seeds may have potential antibacterial activity against multiple antibiotic-resistant bacteria.

Key words: Black cumin, multi-drug resistant bacteria, ethanol extract, chloroform extract.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and the use of medicinal plants, especially in traditional medicine, is currently well acknowledged and established (Kafaru, 1994). Extraction of different bioactive compounds from medicinal plants permits the demonstration of their physiological activity. It also facilitates pharmacological studies leading to synthesis of a more potent drug with reduced toxicity (Ebana et al., 1991; Manna and Abalaka, 2000). Furthermore, the active components of herbal products have the advantage of being combined with many other substances that appear to be inactive and these complementary components give the plant as a whole safety and efficiency much superior to that of its isolated and pure active components (Shariff, 2001). Antimicrobial resistance of pathogens to different drugs is very common, which is a major concern in treatment of various diseases. Inappropriate use of readily available antibiotics, prolonged hospitalization and poor implementation of infection control measures are the main causes of drug resistance. Moreover, powerful drugs against which antimicrobial resistance has not yet been developed are either unavailable or costly. One of the strong solutions of this situation is the use of medicinal plants in healing different infections.

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments (Nascimento et al., 2000; Messina and Messina, 1994). For examples, *Nigella sativa,* which produces black cumin seeds, popularly known in Bengali as Kalogira, is an essential ingredient in the Asian cuisine. It has been used as a medicine and health-promoter for thousands of years. Black cumin seeds were found to exhibit antibacterial

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Table 1. The resistance pattern of the multi-drug resistant (MDR) bacterial isolates against some antibiotics.

MDR bacterial isolates	Resistance pattern
S. aureus ATCC 103207	CAZ, S, BAC, SP, A, V.
B. cereus ATCC 6623	S, BAC, SP, CAZ, TET, NV
B. subtilis ATCC 27853	N, NV, CEP, S, BAC, SP, AMP
E. coli ATCC 12079	S, BAC, SP, V, CEP
Vi. cholerae El Tor	V, NV, SP, BAC, S, AMP
K. pneumoniae ATCC 13883	S, SP, BAC, A, TET, CEP.

AMP = ampicillin (10 μg), A = amoxicillin (10 μg), CAZ = ceftazimide (30 μg), S = streptomycine delet e (30 μg), NV = novobiocin (10 μg), TET = tetracycline (30 μg), BAC = bacitracin (10 μg), CEP = Cephradin (30 μg), SP = spectinomycin (10 μg), V = vancomycin (30 μg), N = nalidixic acid (10 μg).

activity against wide range of gram-positive and gram-negative bacteria (Kumar and Berwal, 1998) and also antiviral activity (Tsai et al., 1985). Cumin seeds are also known to act synergistically with antibiotics, therefore, more dose-response preclinical studies can be done to assess the effectiveness of use of antibiotic/kalogira combination for bacteria that are difficult to eradicate (Gowsala, 2001). So, in this study, the antibacterial activity of the polar and non-polar solvent extracts of cumin seeds was assayed against some pathogenic multi-drug resistant bacteria under laboratory conditions.

MATERIALS AND METHODS

Bacterial strains

Six different multi drug resistant (MDR) bacterial strains were obtained from the stock culture of the Department of Microbiology, University of Dhaka, Bangladesh and listed in Table 1. These strains were grown in nutrient broth (Difco Laboratories, USA) at 37°C and maintained on nutrient agar slants at 4°C.

Black cumin seeds

Black cumin seeds were bought from the spice market and sorted for separation of dirt and unwanted materials. The seeds were dried at 40°C overnight and were ground to powder in a grinder.

Preparation of chloroform and ethanol extracts of black cumin seeds

Chloroform extract: 16.7 g of the dried black cumin seed powder was taken in a 150 ml conical flask and 66.8 ml of chloroform was added to the powder, which was kept at 25°C for 24 h in a dark place. The suspensions were filtrated through a sterile filter paper (Whatman-1) and the filtrate was dried in a beaker at 55°C for two days to get a solid extract. The solid residue was dried again at 55°C overnight to remove residual chloroform.

Ethanol extract: 13.6 g of dried cumin powder was added to 54.4 ml ethanol and kept at 25°C for 24 h. The suspensions were filtrated through a sterile filter paper (Whatman-1). The filtrate was then dried in a beaker at 55°C for two days to get the extract. The residual solid was discarded. All the solvent extracts were weighed in a precision electronic balance. The dried fractions were refrigerated for further study. The final concentration of both chloroform and ethanol extract was fixed at 18 mg/ml.

Test for antibacterial activity

The antibacterial activity of the chloroform and ethanol extracts of black cumin seed samples were evaluated using agar disc-diffusion according to the Kirby Bauer method (Bauer and Thornsberry, 1985) against the food-borne pathogens and spoilage organisms. Seven mm diameter discs were impregnated with 150 μl (at concentration 18 mg/ml) of seed extract and were then dried two days on a clean Petri dish.

The inocula of the test organisms were prepared by transferring 3 to 4 colonies of the cultures (18 h old) into 9 ml of sterile Mueller Hinton Broth (Difco) and incubated at 37°C for 4 to 5 h. The bacterial cultures were adjusted with McFarland turbidity standard (10^8 CFU/ml) and streaked evenly onto the Mueller Hinton agar plate with sterile cotton swab. The seeded plates were left for drying for 3 to 5 min, and the discs were placed on the agar using a sterile forceps and were gently pressed down to ensure contact. Plates were kept at refrigeration temperature for 3 - 4 h for better absorption, during this time microorganisms will not grow but absorption of extracts would take place. Then the plates were incubated in an upright position at 37°C overnight for 24 h. Antibacterial activity was evaluated by measuring the zone of inhibition in mm (including the 7 mm disk) near the agar surface and the results were recorded. A reading of 7 mm meant no zone of inhibition. The end point was taken as complete inhibition of growth as determined by the naked eye. Positive control (chloramphenicol, 30 μg disc) and negative control (discs soaked separately with 95% chloroform or ethanol) were also included for each experiment.

Assay of drug resistance pattern of the test-bacterial strains

The tested bacterial strains were checked for antibiotic sensitivity patterns against different antibiotics using Kirby Bauer method.

Antibacterial activity of different dilutions of ethanol extract

Solutions of ethanol extract were prepared in normal saline at concentrations of 14, 12, 10 and 8 mg/ml. Fifty micro liters of these extracts were soaked on a paper disc and were dried at 30°C for 48 h to remove residual ethanol effects. Mueller-Hinton agar plates were inoculated with the test-bacterial strains. Paper discs were soaked in the ethanol extract and were placed onto the medium as mentioned above.

Determination of minimum inhibitory concentration and minimum lethal concentrations of the ethanol extract

The MIC of the cumin (*N. sativa*) seed extract was determined by tube dilution techniques in Mueller-Hinton broth. The concentration of the ethanol extract used was 4500 to 17.58 μg/ml in Muller Hinton broth by two fold serial dilutions. Approximately $1x\ 10^7$ cfu/ml of inocula of test- bacterial strains were prepared. One ml of the ethanol extract for each concentration was then added to 1 ml of the test-bacterial suspension, making the final reaction volume to 2 ml. A positive and a negative control were also included, where the positive control contained one ml of the extract and the negative control contained no cumin (*N. sativa*) seed extract. The vials were then incubated at 37°C for 24 h. The highest dilution that exhibited

Table 2. Zones of inhibition of cultures of the evaluated bacteria on Muller-Hinton agar plate (MHA).

Bacterial isolates	Diameter of zone of inhibition (mm)		
	Antibiotic (Chloramphenicol 30 µg)	Chloroform extract	Ethanol extract
S. aureus ATCC 103207	17	19	26
B. cereus ATCC 6623	13	16	25
B. subtilis ATCC 27853	21	24	28
E. coli ATCC 12079	16	Resistant	21
Vi. cholerae El Tor	14	20	24
K. pneumoniae ATCC 13883	17	Resistant	Resistant

Table 3. Comparative study of the ethanol extracts at different concentrations with ciprofloxacin 30 µg disc.

Bacterial isolates	Diameter of zone of inhibition of different dilution of ethanol extract in mm				
	14 mg/ml	12 mg/ml	10 mg/ml	8 mg/ml	CIP* 30 µg
S. aureus ATCC 103207	17	14	14	12	20
B. cereus ATCC 6623	16	13	13	11	20
B. subtilis ATCC 27853	19	17	16	16	22
E. coli ATCC 12079	15	15	13	12	19
V. cholerae El Tor	17	15	13	12	18

*CIP means ciprofloxacin antibiotic which has been used as a control.

no visible growth was recorded as the MIC. The vials without growth from the MIC procedure were streaked onto nutrient agar (NA) plates. The plates were then incubated at 37°C for 24 h. The lowest concentration that killed 100% of the test-bacteria (no growth on plate) was recorded as MLC.

RESULTS AND DISCUSSION

Drug resistance pattern of the test-bacterial strains

The bacterial strains were tested for antibiotic sensitivity patterns against different antibiotics. The antimicrobial resistance pattern of the isolates is shown in Table 1. All the bacterial strains were resistant to bacitracin, streptomycin and spectinomycin antibiotics. About 60 to 70% bacteria were found resistant to ceftazimide, ampicillin, nalidixic acid and cephradin. About 20 to 40% bacteria were found resistant to tetracycline, novobiocin, and vancomycin antibiotics.

Sensitivity pattern of the tested bacterial strains against crude extracts of black cumin seeds

The crude chloroform and ethanol extracts of the cumin powder (18 mg/ml) were tested for their antibacterial activity against the tested bacterial strains (Table 2). All the tested strains were sensitive to chloroform extract except *Escherichia coli* ATCC 12079 and *Klebsiella pneumoniae* ATCC 13883, and all the strains were sensitive to ethanol extract except *K. pneumoniae* ATCC 13883. This organism was resistant to both chloroform and ethanol extracts.

Determination of minimum inhibitory concentration and minimum lethal concentration of ethanol extract of black cumin seeds

The MIC and MLC of the ethanol extract against different pathogenic bacteria were determined by tube-dilution method (Figure 1). The test was done in glass vials in Mueller-Hinton broth with ethanol extracts at concentrations ranging from 4500 to17.58 µg/ml. The tube with lowest concentration yielding no visible growth but giving colonies on media is considered as MIC. The lowest concentration of extract that did not yield bacterial growth both in tube and on nutrient agar plate is considered as MLC. Black cumin seed extract is highly effective for *Bacillus subtilis* ATCC 6623 (MIC value 375 µg/ml) followed by *Staphylococcus aureus* (MIC value 1125 µg/ml), *Bacillus cereus* ATCC 6623 (MIC value 1500 µg/ml) and *Vibrio cholerae* El Tor (MIC value 2250 µg/ml) and finally for *E. coli* which achieved lowest MIC value (3000 µg/ml).

In this study, the black cumin seeds, one of the easily

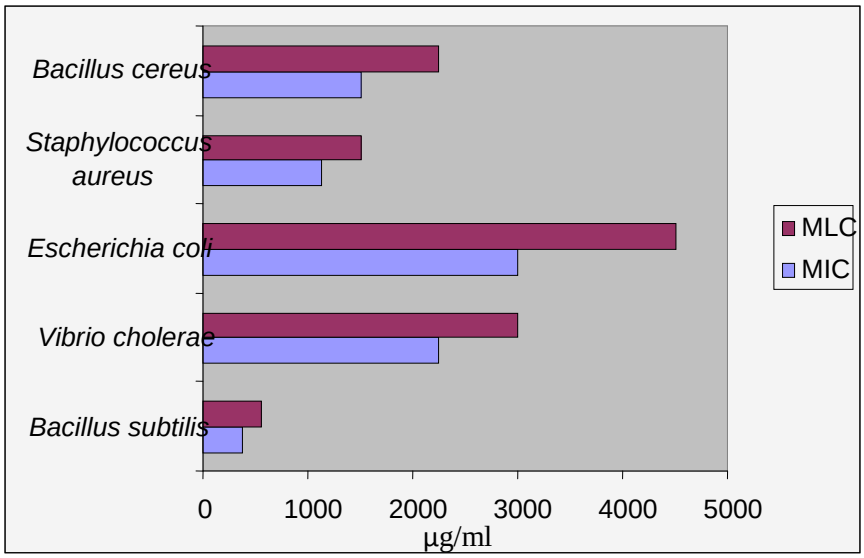

Figure 1. The MICs (minimal inhibitory concentrations) and MLCs (minimal lethal concentrations) of ethanol extracts against MDR (multi drug resistant) isolates.

available herbs in Bangladesh with its known antibacterial activity (Ankri and Mirelman, 1999) were evaluated against some of the common drug resistant bacteria. Unidentified substances from black cumin were extracted using non-polar solvent extraction procedures. All the tested bacterial strains were sensitive to ethanol extract except *K. pneumoniae*. The ethanol extract of black cumin was highly effective against *S. aureus, B. subtilis, E. coli, B. cereus,* and *V. cholerae* El Tor bacteria.

MIC of ethanol extract of black cumin seeds against five bacterial isolates (*S. aureus, B. subtilis, B. cereus, V. cholerae* El Tor and *E. coli*) were determined. Variable MIC values were revealed among the tested strains (Table 3). Highest effectiveness was shown against *B. subtilis* followed by *S. aureus.* On the other hand, the lowest activity of the black cumin seed extract was shown against *E. coli*. Interestingly, the extract could be used against *V. cholerae* bacteria as it showed high susceptibility to this extract. Such inhibitory property of the black cumin seed extract would imply that when administered to cholera patients it may lower the morbidity and mortality rate of the patients, especially for children in remote places.

Also our results showed that *S. aureus*, a common food poisoning organism, was also inhibited. Since *S. aureus* are commonly implicated pyogenic (Rowe et al., 1987; Tanaka et al., 1989), it would be interesting to guess that the extract could be used as an alternative in the treatment of wounds infected with this multi-drug resistant bacterium.

In conclusion, resistance to multiple drugs has become a common feature in which most of the organisms are associated with diarrhea and other enteric diseases (Dhar et al., 1996; Jahan and Hossain,1997; Mamun et al., 2004), urinary tract infection (Chowdhury et al., 1994; Haque et al., 2001), neonatal infection (Bakht et al., 2000; Saha et al., 2003) and wound infection (Rahman et al., 1997; Ahmed et al., 2004; Jahan et al., 2004). In most cases, resistance was found against all commonly prescribed drugs, which is very alarming particularly for a country like Bangladesh, where majority of the population even cannot afford appropriate treatment. Therefore, black cumin seed, which was shown to have antibacterial activity against different multi-drug resistant bacteria, can be used as an alternative and cheap medicine. Further work is needed to improve the extraction procedure of the black cumin seeds and to separate the components of cumin seeds that are responsible for its antibacterial activity.

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