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Antibacterial Activity of *Vitex negundo* L. against a Multidrug Resistant Pathogenic Bacterium

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MAM and GMSU designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MMI and MRN managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this present study is to investigate antibacterial activity of extracts and/or essential oils of *Vitex negundo L.* leaves to an unknown pathogenic bacterium have been resistant to various groups of antibiotics
Study Design: The study was design followed by previously studied and manuals of antimicrobial susceptibility test.
Methodology: For antimicrobial susceptibility test disks diffusion method were used according to the guideline of European Committee on Antimicrobial Susceptibility Testing followed by McFarland standards. Various groups of antibiotics were used for conforming resistant of this unknown pathogen. The zone of inhibition and MIC was compared and measures with National Committee for Clinical Laboratory Standards (NCCLS) guidelines and reference data of previously studied.
Results: According to NCCLS and data to our study revealed that UB201201 (unknown) was a

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multidrug resistant bacterium. Molecular identification discloses the bacterium were *Bacillus cereus* group. All extracts of *Vitex negundo L.* leaves (ethanol, chloroform, ethyl acetate and hexane) and essential oils were responsible for antibacterial susceptibility with inhibition zone of 29±0.7, 9±0.5, 8±0.3, 12±0.7, and 25±0.2 mm respectively.

Conclusion: This study signifies ethanolic extract of *Vitex negundo* L. leaves as moderate antimicrobials and can be used for pharmaceutical and medicinal purpose. Therefore, isolation and identification of bioactive compounds from this plant will be an interest for human being.

Keywords: Vitex negundo L.; multidrug-resistant bacteria; Bacillus cereus; antimicrobial agents; antibiotics; bioassay.

1. INTRODUCTION

In recent years, the emergence of pathogenic microorganisms acquired resistance to a wide range of formerly efficacious antibiotics has become a major cause of concern both in hospital settings and in the community [1,2]. The antibiotic-resistant bacteria are germs that are not killed by commonly used antibiotics and when bacteria are exposed to the same antibiotics over and over, the bacteria can change and are no longer affected by the drug. Moreover, the risk of transferring antimicrobial drug resistance to nonresistant bacteria and the propagation of multidrug-resistant (MDR) bacteria from agricultural to clinical and/or community-associated settings are being debated by research, regulatory, and health authorities [3,4]. Despite emphasis being put in research of synthetic drugs, a certain interest in medicinal plants has been reborn, in part due to the fact that a lot of synthetic drugs are potentially toxic and are not free of side effects on the host [5]. Because of the side effects and the resistance that pathogenic microorganisms build against antibiotics, much attention has been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine. *Vitex negundo* L. (*Verbenaceae*), is an important medicinal plant found throughout Bangladesh (known as Nishinda) using as an important source of natural drugs from ancient time (Ayurvedic and Unani Systems of Medicine) and a number of pharmacological and medicinal activities and properties have been attributed toit by several studies [6-44].The aim of the present study is to investigate antibacterial activity of extracts and/or essential oils of *Vitex negundo L.* leaves to an unknown pathogenic bacterium have been resistant to various groups of antibiotics.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria and Antimicrobial Bioassay

Bacteria were isolated from an eighteen years diarrheal patient in Rajshahi Medical College Hospital, Rajshahi and subjected to pure culture by using nutrient agar media (Merck Ltd. Germany). The antibacterial activity of the test samples was tested by disc diffusion method according to Uropean Committee on Antimicrobial Susceptibility Testing (UCAST, Version 3, 2013) [45]. Bacterial strains grown on nutrient agar at 37°C for 24 hours were adjusted to the turbidity of the 0.5 McFarland standards (10^6colony forming units/mL). Then, the standard inoculums were spread by sterile cotton on the surfaces of nutrient agar prepared for growth of the bacteria. The disks (6mm in diameter) were impregnated with 10 µL at various concentrations of the extracts and placed on the inoculated media. The petri dishes incubated overnight at 37°C for bacterial growth and inhibition zone were examined carefully. Commercial Ampicillin, Streptomycin, Trimethoprime, Ciprofloxacin, Azithromycin and Tetracycline discs were used as standard antibiotics. The antibacterial activity was determined by measuring the diameter of zone of inhibition by millimeter scale.

2.2 Collection and Extraction of Plant Material

The mature fresh plant leaves of *Vitex negundo* L. were collected from the region of kushtia, Bangladesh and washed properly with sterile distilled water and dried at room temperature. The dried powdered was prepared using blender machine. Shaded dry and crashed leaves (150 gm) were subjected to 1000 ml borosilicate glass vessel alone with methanol, chloroform, ethyl acetate and hexane (500 ml of each) as solvents. After 48h the extracts was filtrated and solvents

were then evaporated using rotary evaporator until a concentrated extracts was obtained. Crude extracts were obtained kept at 4ºC until further assay. Water distillation method was used for extraction of essential oil.

2.3 Molecular Identification of the Bacteria

2.3.1 16S rDNA extraction and amplification from isolated bacteria

For molecular analysis, DNA was extracted from isolated colonies by alkaline lysis. 200 µL of culture was suspended in an Eppendorf tube with 50 µL of lysis buffer (2.5 ml 10% sodium dodecyl sulfate, 5 ml 1 M NaOH, 92.5 ml MilliQ water). After 15 min at 95°C and the tube was centrifuged for 5 min at 13,000 x g, and the supernatant was transferred to a new tube to add 90 µL MilliQ water. Extracted DNAs were stored at -20°C for further molecular analyses. For amplification of 16SrDNA from cultural bacteria PCR was performed in a final volume of 25 µl containing buffer 10X, 1.0 unit of *Taq* DNA polymerase (Amersham Biosciences), 0.2 mM each of dNTPs, 200 nM of each primer 63F 5'CAGGCCTAACACATGCAAGTC [46] and 1389R 5'ACGGGCGGTGTGTACAAG [47] and 50 ng template DNA. The thermal cycler (Bio Rad I Cycler 170-8740) was programmed for the initial denaturation step (94°C) of 5 min, followed by 44 cycles of 1 min denaturation along with 1 min primer annealing (37°C) and 2 min primer extension (72°C), followed by the 7 min primer extension (72°C) step. The amplified DNA was visualized by gel electrophoresis. After amplification of 16S r DNA from isolated bacteria were sequenced by automated DNA sequencer.

2.3.2 16S rDNA sequence analysis and molecular characterization of bacteria

The 16S rDNA sequence of UB201201 was used for taxonomical identification and phylogeny analysis. In taxonomical terms, molecular characterizations of the concerned isolates by 16S rDNA sequencing focus on following facts [48] as: 1. Domain Name → Phylum Name → Class Name → Order Name → Family Name → Genus Name → Species Name → Strain Name/Number (Sub-Species level), 2. Retrieving Accession Number from EMBL/GenBank/DDBJ, and 3. Phylogeny Analysis by Constructing Phylogenetic Tree. The bacterial 16S rDNA partial sequence generated in this study was deposited in the International Nucleotide Sequence Databases (INSD) in EMBL/GenBank/DDBJ nucleotide sequence data libraries and its accession number, nomenclature and taxonomical identifications (Table 3) were obtained through the analysis of concerned sequences in the GenBank database, EMBL Nucleotide Sequence Database and DDBJby using the algorithm BLASTN [49]. Phylogeny.fr [50] was used to construct Maximum-likelihood Phylogenetic Trees (PhyML) with our concerned sequence (Fig. 1).

3. RESULTS

3.1 Bacterial Susceptibility Tests

The bacterium was resistant to antibiotics testified by the pathology lab Rajshahi Medical College Hospital and further test were done in Islamic University supported lab. Commercial Ampicillin, Streptomycin, Trimethoprime, Ciprofloxacin, Azithromycin and Tetracycline discs were used as standard antibiotics to test bacterial susceptibility (Table 1). Bacterial susceptibility test was also done by essential oil and different solvent extracts of *Vitex negundo* L. leaves (Table 2). The antibacterial activity was determined by measuring the diameter of zone of inhibition by millimeter scale.

3.2 Molecular Characterization of the Bacterium

The accession number, nomenclature and taxonomical identifications of UB 201201 were obtained through 16S rRNA gene analysis from International Nucleotide Sequence Databases (INSD) (Table 3) and Phylogeny.fr was used to construct Maximum-likelihood Phylogenetic Tree (Fig. 1). The molecular test identified it as *Bacillus cereus* strain mmm86 (AB709908.1) in *Bacillus cereus* group.

4. DISCUSSION AND CONCLUSION

The overuse of antibiotics has become a major problem for the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms in Bangladesh. Both gram-positive and gram-negative bacteria pathogens have shown a remarkable ability to develop resistance to antimicrobial agents which have forced clinicians to seek alternative treatments for patients with serious gram-positive and gram-negative infections. Illnesses due to multidrug-resistant gram-negative microorganisms pose an

important clinical problem, resulting in significant morbidity and mortality worldwide [51,52]. In this study, the Table 1 shows that the bacterial strains UB201201 are multidrug resistant bacteria.

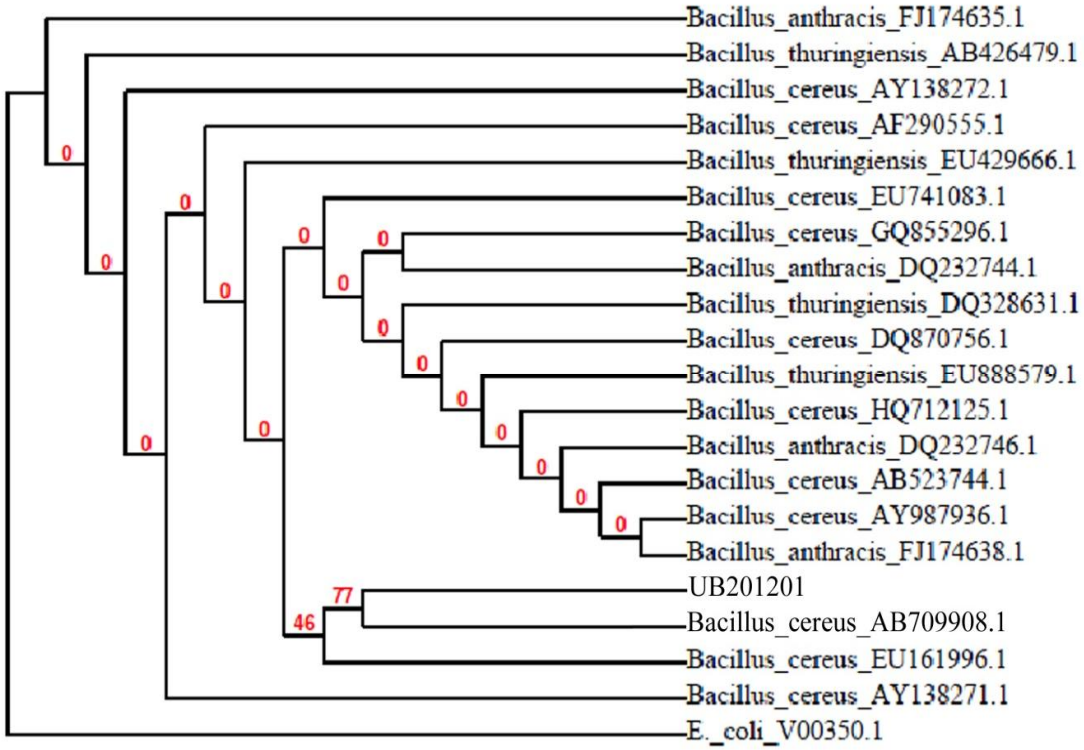

Fig. 1. Phylogenetic tree representing pathogenic bacteria of *Bacillus cereus* group

The phylogenetic tree is shown in cladogram (ignoring branch lengths). bootstrap values (100 replications), branch support values greater than 50% were considered significant while branches are collapsed having branch support value smaller than 50. E. coli is used as outgrowth. All bacteria shown here are close relatives of the UB201201 (most closely related to Bacillus cereus AB709908.1) and they are classified as under Bacillus cereus group except the E. coli

Table 1. Bacterial susceptibility test against various antibiotics

Antibiotics samples	MIC (µg/ml)	Number of test sample (UB201201)	Average zone of Inhibition (mm)	Interpretation (%)	
				S	R
β-Lactams Ampicillin	35	20	15	-	100%
Aminoglycosides Streptomycin	20	20	12	-	100%
Sulfonamides Trimethoprim	30	20	13	-	100%
Quinolones Ciprofloxacin	10	20	10	-	100%
Macrolide Azithromycin	5	20	14	-	100%
Tetracycline Tetracycline	20	20	17	45%	50%

Table 2. Antibacterial activities of various extracts of *Vitex negundo L.* leaves

Extracts	Zone of inhibition (mm)	Minimum Inhibitory Concentration (MIC)	Bacterial susceptibility
	UB201201		
Ethanol	29±0.7	128 mg/ml	Susceptible
Chloroform	9±0.5	128 mg/ml	Susceptible
Ethyl Acetate	8±0.3	128 mg/ml	Susceptible
Hexane	12±0.7	256 mg/ml	Susceptible
Essential Oil	25±0.2	50 v/v	Susceptible

Table 3. Molecular characterization of the concerned bacterium

Features	UB 201201
Molecular Type	Genomic DNA
Topology	Linear
Gene	16S rRNA
Product	16S ribosomal RNA
Accession Number	AB709908.1
Identity/Similarity (%)	100
Closest Relatives	*Bacillus cereus*
Organism	*Bacillus cereus*
Strain	mmm86
Isolation Source	*Homo sapiens*18 years old male patient
Taxonomic Division	PRO
Taxonomy EMBL	Bacteria Firmicutes Bacillales Bacillaceae Bacillus cereus group
Taxonomy Green Gene	k_Bacteria p_Firmicutes c_Bacilli o_Bacillales f_Bacillaceae g_Bacillus s_Bacillus cereus
Taxonomy SILVA	Bacteria Firmicute Bacilli Bacillales Bacillace Bacillus
Taxon	Taxon:1396

The 16S rDNA partial sequences of the unknown bacterial strain UB201201 generated in the present study were deposited in the International Nucleotide Sequence Databases (INSD) and its accession numbers, nomenclature and taxonomical identification were obtained through the analysis of concerned sequences in the GenBank Database (Table 3). It is mentionable that thecriteria for identification to the species level were defined as 16S rDNA sequence similarity of 99% with that of the prototype strain sequence in GenBank [53]. The similarity sequence scores of UB201201 in comparison to those sequences deposited in GenBank and /or EMBL Nucleotide Sequence Database were 100% suggesting that this is the close relative of *Bacillus cereus* and identified as *Bacillus cereus* strain mmm86 (AB709908.1). The taxonomical and nomenclatural identifications reported by Taxonomy EMBL classify *Bacillus cereus* strainmmm86 as Bacteria→ Firmicutes→ Bacillales→ Bacillaceae→ Bacillus→ *Bacillus*

cereus group. The phylogenetic analysis (Fig. 1) has shown, in comparison with sequences of pathogenic bacteria, that the *Bacillus cereus* strainmmm86 is a pathogenic bacterium under *Bacillus cereus* group. In practical case, according to Vilas-Boas et al. [54], the *Bacillus cereus* group contains three species as *Bacillus cereus, Bacillus anthracis and Bacillus thuringiensis*, of them *Bacillus* cereus and *Bacillus anthracis*are important pathogens of mammals, including humans, and *Bacillus thuringiensis* is extensively used in the biological control of insects. The antibiotic susceptibility test of *Bacillus cereus* strain mmm86 to various group of antibiotics shown in Table 1. The zones of inhibition were compared and measures with National Committee for Clinical Laboratory Standards (NCCLS) guidelines [55]. MIC value were also compared with the MIC range of reference value have been previously reported and confirm resistant to these antibiotics [56-58].

Bacillus cereus has emerged as one of the most virulent bacteria and the etiological agent of two distinct food poisoning syndromes [59], as the diarrhoeal-type and the emetic-type. *Bacillus cereus* infections occur mainly, though not exclusively, in persons predisposed by neoplastic disease, immunosuppression, alcoholism and other drug abuse, or some other underlying condition, and fatalities occasionally result. Reported conditions include bacteremia, septicemia, fulminant sepsis with hemolysis, meningitis, brain hemorrhage, ventricular shunt infections, infections associated with central venous catheters, endocarditis, pseudomembranous tracheobronchitis, endophthalmitis, pneumonia, periodontitis, empyema, pleurisy, lung abscess, brain abscess, osteomyelitis, salpingitis, urinary tract infection, dermatolymphangioadenitis associated with filarial lymphedema, and primary cutaneous infections [60,61]. It is a well-recognized agent of mastitis and abortion in cattle, and can cause these conditions in other livestock [62]. Nevertheless, there is limited information available with respect to its antimicrobial agent susceptibility. Published data suggest that *Bacillus cereus* isolates are susceptible in vitro to gentamicin, vancomycin, clindamycin, chloramphenicol, and erythromycin [63,64]. Resistant to penicillin, ampicillin, trimethoprim andcephalosporin have also been reported [65]. The most possible reason behind multidrug resistances of *Bacillus cereus* that it produces a broad spectrumβ-lactamase which hydrolyzes β-lactam rings of antibiotics and another important fact is that they are spore-forming bacteria allowing them to survive in different types of adverse environments.

Vitex negundo L. possesses antibacterial activity against *Escherichia coli*, *Klebsiella aerogenes*, *Proteus vulgaris*, and *Pseudomonas aerogenes* [66]. The Methanol and/or ethanol extracts of *Vitex negundo* L. leaves have been reported to have prominent zone of inhibition against *Bacillus subtilis, Staphylococcus aureus, Micrococcus pyogenes, Pseudomonas aeruginosa, E. coli, Bacillus subtilis, Bacillus megaterium, Salmonella typhi, Vibrio mimicus, C. albicansetc* [67-74]. In this study, we found that all extracts (ethanol, chloroform, ethyl acetate and hexane) and essential oils have antibacterial activities represents inhibition zone of 29±0.7, 9±0.5, 8±0.3, 12±0.7, and 25±0.2 mm respectively (Table 2). Among them ethanol extract reflect more bio-efficacy and antibacterial potency (inhibitory zone 29±0.7 mm) against the identified bacterium depicting that it contains such bioactive compounds and/or phytochemical constituents that can retard the bacterial growth or kill *Bacillus cereus*strainmmm86. So, we can say that the extractability of bioactive compounds is greatly influenced by polar compounds. Thus, the ethanol extracts of *Vitex negundo* L. contains such bioactive compound(s) that have the ability to inhibit the growth or to kill the concerned multi-drug resistant bacterium (*Bacillus cereus* strain mmm86) and can be used for pharmaceutical and medicinal purpose. How extracts of *Vitex negundo* L. be responsible for antibacterial activity against multi-drug resistant bacterium is unclear but it may be due to the presence of some compounds like β–Caryophyllene, Ag (Silver) nanoparticles, betulinicacidsetc. have been previously reported in several study [75-79]. Therefore, this study may be taken into consideration for future therapeutic and drug development purpose for which further research is needed.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

No conflicts of interest have been disclosed with the submission of this paper.

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