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Article

Medicinal plants available in local markets contaminated with human pathogenic bacteria - *Bacillus cereus* and *Pseudomonas gessardii*

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Abstract: Medicinal plants are known to be a potential source of therapeutic aids. Different uses of medicinal plants have attained a powerful role in health system throughout the world. To study the microbial risk factor associated with medicinal plants, medicinal plant and part of plant samples (10 from each location) were collected from three different local markets (Nabinagar, Amin Bazar, and Savar) of Savar area, Dhaka, Bangladesh. A total number of fifty five (55) bacterial isolates have been isolated through their growth on Nutrient Agar media. Out of 55 isolates 31 isolates (56%) were identified as *Bacillus cereus* and 7 isolates (13%) were identified as *Pseudomonas* sp. through different morphological and biochemical tests. The CFU value of different samples revealed that all the values were higher than the accepted value (recommended by WHO, 2007) of 10^5 CFU/g except *Phyllanthus emblica*. The PCR and 16S rRNA sequencing confirmed the identification similarity values among two isolates of *B. cereus* and *Pseudomonas gessardii*. Both *B. cereus* and *Pseudomonas gessardii* showed sensitivity to the antibiotics Kanamycin, Gentamycin, Tigecycline, Ciprofloxacin and Amikacin where resistance to the antibiotics Amoxycilin, Aztreonam, Penicillin-G, Cefixime, Cefotaxime, Cefepime, Ceftriaxone, Meropenem, Cotrimoxazole and Ceftazidime. Therefore, quality assessment of herbal medicines should be ensured to make them safer for human consumption.

Keywords: prevalence; herbal medicine; commercial market; 16S rRNA; disease causing bacteria

1. Introduction

Medicinal plants are defined as those plants which are commonly used in preventing and treating specific diseases and ailments (Schulz *et al*., 2001). These plants may be either "Wild plant species" – plants growing in semi natural or natural ecosystems spontaneously in self-maintaining populations or "Domesticated plants species" which arise through different human actions like selection or breeding and depend for their existence on management practices (Calixto, 2000). Different medicinal plants have been discovered and used in traditional practices since prehistoric times. It has been known as the first art of treatment to human kind. Although medicinal plants were used as dietary supplements or therapeutic use dates back beyond recorded history, it has increased substantially in the past decades (WHO, 2002). Now-a-days, the uses of medicinal plants have achieved a major role in health system throughout the world. According to the World Health Organization, about 70-80% of the World's population, particularly in developing countries relies on different non-conventional medicines for their primary healthcare (Akerele, 1993).

Handling, processing and preserving steps of these medicinal plants may be contaminated with bacterial biota. Medicinal herbs frequently harbor lots of microorganisms originating primarily from the soil and these microbes

adhere normally to roots, stems, leaves, flowers and seeds of plants (Adeleye *et al*., 2011). Contaminating microbes and their toxins lead to disease which makes them hazardous for human consumption (Govender *et al*., 2006). *Bacillus cereus* is recognized for having potential pathogenicity and has been implicated in food poisoning (Kunene *et al*., 1999). It is a gram-positive spore-forming bacterium which causes foodborne disease and is widespread in foods and nature (Marrollo, 2016). *B. cereus* spores are hydrophobic in nature and can adhere to surfaces forming biofilm, which results in the recurrent contamination. Food poisoning strains of *B. cereus* can produce four different enterotoxins viz. two enterotoxic proteins and two proteinous complexes such as nonhemolytic enterotoxin and hemolysin BL (HBL) (Lund *et al*., 2000). Two kinds of food-borne diseases are caused by *Bacillus cereus*; one is emetic intoxication (vomiting) caused by ingestion of cereulide toxin produced in foods and another is diarrheal infection caused by bacterial cells/spores ingestion that is capable of producing enterotoxin in small intestine (Pal *et al*., 2014a). So, it is an important foodborne opportunistic pathogen and *B. cereus* is suggested to be controlled (Yang *et al*., 2017). On the other hand, *Pseudomonas gessardii* is a fluorescent, Gram-negative, rod-shaped bacterium (*Verhille et al., 1999*) which has been placed in the *P. fluorescens* group based on 16S rRNA analysis (*Anzai et al., 2000). Pseudomonas fluorescens* are opportunistic pathogens and cause unusual diseases in humans and usually affect patients with compromised immune systems (e.g., patients on cancer treatment).

Inevitably, the presence of pathogenic bacteria in medicinal plants depends on a number of environmental factors which exerts a significant impact on the inclusive quality of herbal preparations and products. Among these, one of the most important contributing factors for their growth is increase of temperatures and moisture (Hell and Mutegi, 2011). During drying process, pathogens can be sub-lethally injured and the damage to the cell is minimal, so the cell can recover. These sub-lethally injured microorganisms have been reported to stay alive up to two years (Schottroff *et al*., 2018). The risk of microbial contamination often increases in areas where harvesting is done during the rainy season or during the period of high temperatures and humidity (WHO, 2003). The ways by which medicinal plants are transported to markets further enhances the risk of microbiological cross contamination (Katerere *et al*., 2008).

Therefore, risk assessment through determination of the microbiological load of different medicinal plants has been an important subject in the establishment of modern Hazard Analysis and Critical Control Point (HACCP) schemes. In Bangladesh, a large population of rural areas uses different medicinal plant and plant parts for treating different diseases. Instead of getting well, they often suffer from various foodborne diseases using these medicinal plants because of the unhygienic condition during preparation and handling process of these herbal medicines. So, it is necessary to evaluate the occurrence and identification of potentially pathogenic bacteria and their antimicrobial susceptibility pattern isolated from different herbal medicines sold in local markets of Bangladesh. Therefore, this study was designed at identifying the presence of pathogenic bacteria like *Bacillus cereus* and *Pseudomonas gessardii* present in medicinal plants of the studied areas using molecular based technique.

2. Materials and Methods

2.1. Sample collection

A total of 30 samples (10 from each location) of ten medicinal plants (root, leaves, fruit, seed etc.) such as *Plantago psyllium*, *Terminalia bellirica*, *Terminalia chebula*, *Phyllanthus emblica*, *Ocimum basilicum*, *Glycyrrhiza glabra*, *Withania somnifera*, *Andrographis panicula*, *Bombax ceiba* and Trifola (a mixture of *Terminalia bellirica*, *Terminalia chebula* and *Phyllanthus emblica* fruit) were collected from three local markets (Nabinagar, Amin Bazar, Savar) at Savar area of Dhaka, Bangladesh. All of these samples were sold as ready-to-eat for medicinal purposes; further processing is not required for consumption. After collection, the samples were brought to the laboratory and opened in the laminar air flow cabinet. They were preserved at 4ºC until further study.

2.2. Isolation of bacteria

Bacterial isolation and enumeration was conducted by spread plate method on Nutrient Agar media (NA) plate (Eklund and Lankford, 1967). The microbiological hygiene and safety condition were then assayed using the techniques recommended by International Commission on Microbiological Specifications for Foods (ICMSF, 2005). 0.1 ml sample of each dilution was taken onto sterile petridish and spread evenly on the solid nutrient agar media and incubated for 24 hours at 37°C. After the incubation period, plates which have discrete colonies were used for counting. After counting, distinct bacterial colonies were immediately isolated. Based on specific colony morphology, further selection has been made and the isolates were purified by repeated streaking. Then they were stored at 4°C in NA slants for further analysis (Afrin *et al*., 2019).

2.3. Microbial load determination

Total number of discrete colonies from 3 different plates of each sample were counted after incubation and used for microbial load determination. Microbial load is determined using the following equation -Number of CFU/ g = Number of Colony/ (Volume plated in ml ×total dilution used) (Afrin *et al*., 2018). Isolated colonies were counted in colony formation unit (CFU per gram).

2.4. Identification of the isolates

Selected and isolated bacterial colonies were examined to study various morphological characters *viz.* form, color, margin, elevation, surface, optical characters etc. as stated by Eklund and Lankford (1967). Then the colonies were cultured on specific selective media of *Bacillus* sp. and *Pseudomonas* sp. *B. cereus* selective agar (BCSA) base which contained $MgSO_4$, KH_2PO_4, NaCl, peptic digest of animal tissue, sodium pyruvate, mannitol, Na_2HPO_4 and bromothymol blue supplemented with polymyxin B sulfate and sterile egg yolk was used for *Bacillus cereus* identification and Cetrimide agar containing Pancreatic Digest of Gelatin, Potassium Sulfate, Magnesium Chloride, Cetyltrimethyl ammonium Bromide and Glycerin was used for selective isolation of *Pseudomonas* sp. A number of biochemical tests (Fermentation test, Indole test, Motility test, MR-VP test, Catalase test, Citrate utilization test etc.) were also performed. Gram staining and endospore staining were also performed. Casein test and Starch hydrolysis test were used for the presence of protease and amylase enzyme. Result of the morphological, physiological and biochemical test of selected isolates was analyzed following the Bergey's Manual of Determinative Bacteriology (Halt, 1998).

2.5. 16S rRNA gene amplification by PCR (Polymerase Chain Reaction)

Selected isolates were subjected to PCR of partial sequence of 16S rRNA of the bacterium for identification. A commercial DNA extraction kit (Promega, USA) was used according to the supplier's instruction for DNA isolation. Amplification of the partial sequence of 16S rRNA gene was done with universal primer 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) and 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) in present investigation (Lane, 1991). A thermal cycler naming Applied Biosystems 2720 was used for DNA amplification using the following conditions: 35 cycles, preheating at 95ºC for 5 minutes, denaturation at 95ºC for 30 seconds, annealing at 57ºC for 30 seconds and extension at 72ºC for 2 minutes. The amplification product was analyzed by gel electrophoresis in the agarose gel (1%) with buffer solution (TBE buffer). Then they were stained with 0.1% ethidium bromide and illuminated under UV light.

The PCR products of ~1470bp were then sequenced by commercial service provider naming first BASE Laboratories Sdn Bhd of Malaysia. The sequences were analyzed. Finally, identification of bacterial isolates was accomplished by comparing the sequences with sequences available in public databases of NCBI Blast (*Basic Local Alignment Search Tool*) using BLAST search engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi). MEGA version 6 has been used for the multiple sequence alignment and phylogenetic tree (Tamura *et al*., 2004). The phylogenetic trees of the isolates were produced using currently obtained sequence data and retrieving sequence of the NCBI database.

2.6. Susceptibility of bacterial isolates to different antibiotics

The sensitivity of *B. cereus* and *Pseudomonas gessardii* (1 isolate from each location) to fifteen antibiotics namely (Kanamycin, Amoxycilin, Aztreonam, Penicillin-G, Cefixime, Gentamycin, Cefotaxime, Cefepime, Tigecycline, Ceftriaxone, Ciprofloxacin, Meropenem, Cotrimoxazole, Amikacin, Ceftazidime) was studied by using Standard Kirby-Bauer disk diffusion method (Yu *et al*., 2019). These fifteen antibiotics were chosen due to their wide spectrum activity in opposition to both Gram-negative as well as Gram-positive pathogenic bacteria. The *B. cereus* and *Pseudomonas sp.* isolates from preserved slant were inoculated into NA broth medium and allowed to grow a specified density at 37°C for 24 hours. A sterile swab is dipped in the liquid culture. The swab is streaked evenly over a plate of sterile Mueller-Hinton agar. Different antimicrobial disks were placed on the surface of the MH agar plates with sterile forceps after drying of the inoculum. Then the plates were incubated at 35 ± 2°C for 24 hours, and inhibition zones were measured. All results were recorded appropriately and interpreted using the National Committee for Clinical Laboratory Standards interpretation chart (CLSI, 2008).

The isolates were classified and interpreted as resistant (R), susceptible (S) or intermediate (I) according to CLSI and zone diameter interpretation criteria for *B. cereus* and *Pseudomonas gessardii.*

3. Results

3.1. Microbial load determination

In this study, the average microbial load of different medicinal plant samples ranged from $6.57x10^4$ CFU/g to $1.65x10^{15}$ CFU/g (Table 1). There was no significant difference among the CFU of different location's medicinal plant samples.

3.2. Morphological and biochemical characterization

A total number of 55 isolates were isolated from thirty different medicinal plant and plant parts (seed, leaves, fruits) in the present study. Out of 55 isolates 31 isolates (56%) were identified as *Bacillus cereus* based on their growth on selective media. Biochemical and morphological characterization of bacterial isolates indicated that all of these 31 isolates were motile and rod shaped, positive for gram staining, citrate utilization, and endospore staining. All these 31 isolates were negative for indole, urease and mannitol test and gave positive results for starch hydrolysis and casein test indicating the presence of amylase and protease enzyme. On the other hand, out of 55 isolates 7 isolates (13%) were identified as *Pseudomonas* sp. These 7 isolates were motile and rod shaped, negative for gram staining, positive for catalase, oxidase, citrate utilization, casein and starch hydrolysis test. While, all these 7 isolates were negative for MR, VP, indol and urease test (Table 1). Among different medicinal plant samples, abundance of *Bacillus cereus* was highest (80%) in *Withania somnifera* and lowest (25%) was in *Phyllanthus emblica*. In case of *Pseudomonas* sp., abundance of *Pseudomonas* sp. was highest (45%) in *Plantago psyllium* and lowest was (0%) in *Terminalia chebula* and *Bombax ceiba*.

Table 1. Bacterial colony forming unit (CFU) of different analyzed samples of medicinal plant collected from Savar area.

Medicinal plant samples	Used part	Average CFU/g (mean ± SD)	*Bacillus cereus*	*Pseudomonas* sp.	Other bacteria	Accepted limit recommended by WHO (CFU/g)
Plantago psyllium (Isupguler vushi)	Husk	$5.4x10^5$± 1.0	27%	45%	28%	10^5
Ocimum basilicum (Tokma)	Seed	$2.97x10^{14}$±0.58	45%	18%	37%	
Terminalia bellirica (Bohera)	Fruit	$7.83x10^6$ ±1.53	44%	11%	45%	
Terminalia chebula (Horitoki)	Fruit	$8.93x10^6$± 0.58	57%	0%	43%	
Phyllanthus emblica (Amloki)	Fruit	$6.57x10^4$ ±0.58	25%	13%	62%	
Glycyrrhiza glabra (Yasthimadhu)	Root	$4.1x10^{14}$± 1.0	56%	11%	33%	
Withania somnifera (Aswagandha)	Root	$4.7x10^5$± 1.53	80%	7%	13%	
Andrographis paniculata (Kalomegh)	leaves	$3x10^5$ ± 1.15	75%	8%	17%	
Bombax ceiba (shimul mul)	Root	$7.33x10^{14}$±1.53	57%	0%	43%	
Trifola (mixture of *Terminalia bellirica*, *Terminalia chebula* and *Phyllanthus emblica*)	Mixture of fruit powder	$1.65x10^{15}$±1.53	55%	9%	36%	

CFU presented as per gram (The limit of microbial contamination for herbal medicinal products suggested in European Pharmacopoeia [European Pharmacopoeia, 2007] is 1. total aerobic bacteria should be < (10^7 CFU/g) to samples to which boiling water is added before use, 2. total aerobic bacteria < (10^5CFU/g) to samples to which boiling water is not added before use, 3. Enterobacteria and other Gram-negative bacteria should be < (10^3CFU/g) where *Salmonella* sp. and *E. coli* should be absent).

3.3. Molecular characterization

Two bacterial isolates from enrichment cultures were isolated and maintained at 30°C for 24 hours. Then DNA samples of the isolates were visualized after PCR which was ~1470 base pair. Purified PCR products were subjected to Sanger sequencing and DNA sequences of *Bacillus cereus* JUBR1 (MH737743) and *Pseudomonas gessardii* JUBR2 (MH737772) were matched with NCBI database (Figures 1 and 2).

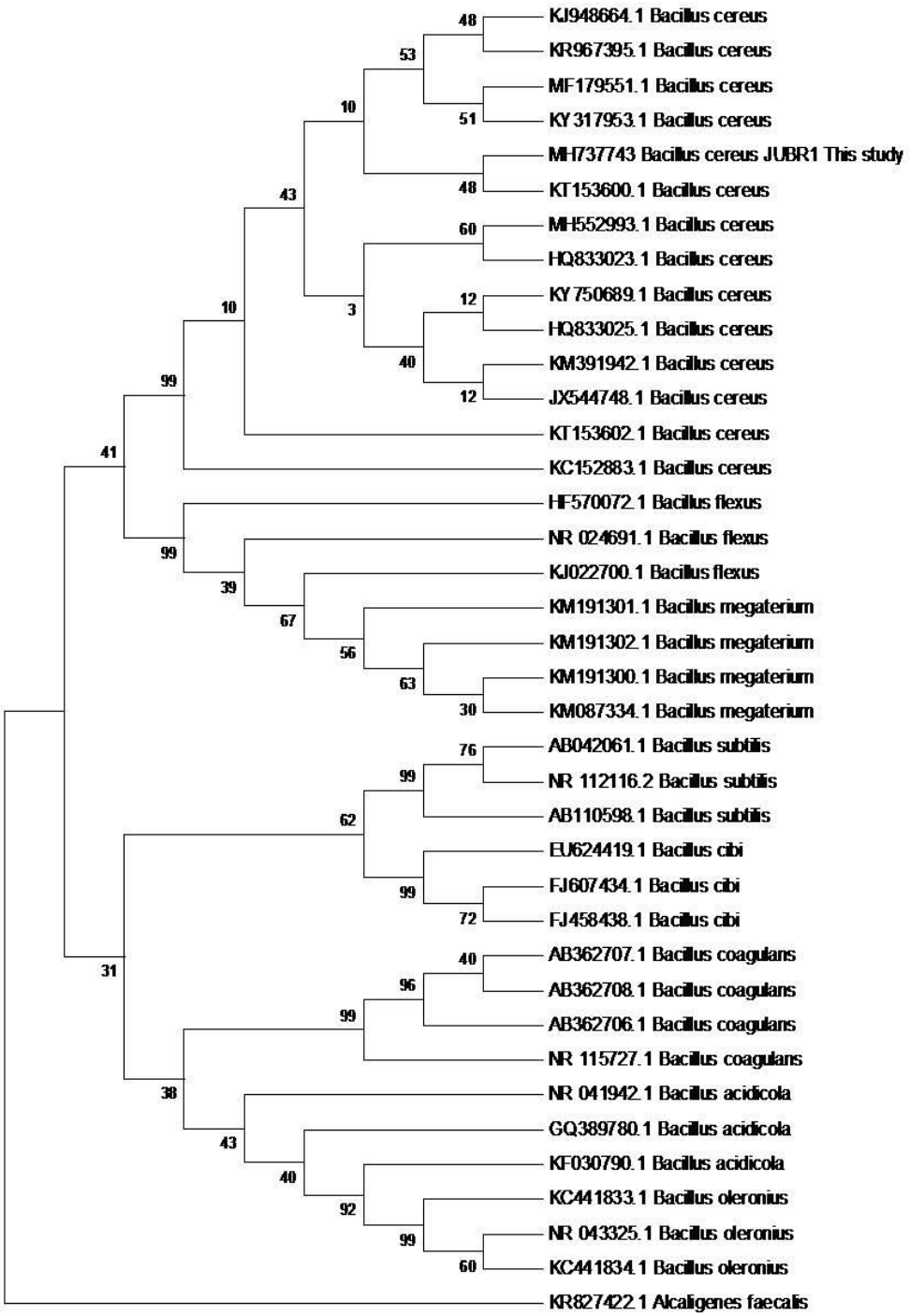

Figure 1. Phylogenetic tree (NJ) of *Bacillus cereus*.

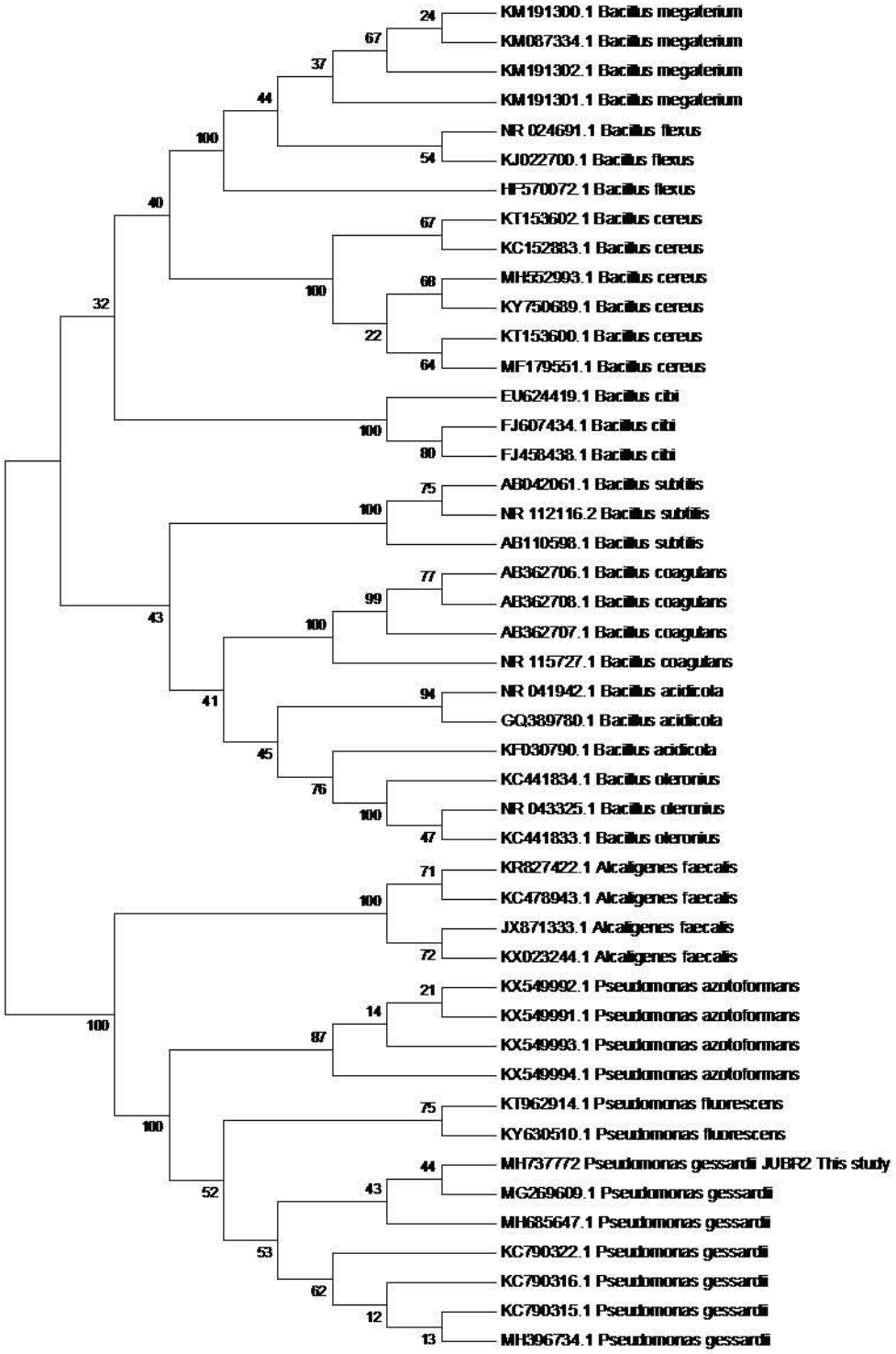

Figure 2. Phylogenetic tree (ML) of *Pseudomonas gessardii*.

3.4. Antibiotic sensitivity

The identified bacteria were assessed to know the sensitivity of the bacteria against the antibiotics Kanamycin, Amoxycilin, Aztreonam, Penicillin-G, Cefixime, Gentamycin, Cefotaxime, Cefepime, Tigecycline, Ceftriaxone, Ciprofloxacin, Meropenem, Cotrimoxazole, Amikacin, Ceftazidime (Table 2).

Table 2. Antibiotic sensitivity pattern of *Bacillus cereus* and *Pseudomonas gessardii* against different antibiotics.

Antibiotics	Bacterial isolates		% resistance
	Bacillus cereus	***Pseudomonas gessardii***	
Kanamycin 30 (K 30)	S	S	00.0
Aztreonam 30 (ATM 30))	R	R	100.0
Penicillin-G 10 (P 10)	R	R	100.0
Cefixime – 5 (CFM 5)	R	R	100.0
Gentamycin – 10 (CN 10)	S	S	00.0
Cefotaxime -30 (CTX 30)	R	R	100.0
Cefepime – 30 (FEP 30)	R	R	100.0
Tigecycline – 15 (TGC 15)	S	S	00.0
Ceftriaxone – 30 (CRO 30)	R	R	100.0
Ciprofloxacin – 5 (CIP 5)	S	S	00.0
Meropenem – 10 (MEM 10)	R	R	100.0
Cotrimoxazole – 25 (SXT 25)	R	R	100.0
Amoxycilin – 30 (AMC 30)	R	R	100.0
Amikacin – 30 (AK 30)	S	S	00.0
Ceftazidime – 30 (CAZ 30)	R	R	100.0

*S = Susceptible, R = Resistant

4. Discussions

Medicinal plants possibly associated with a wide variety of microbial contaminants represented by virus, bacteria and fungi. In the present study, microbial load of a number of medicinal plant samples was determined and in almost all the samples, the CFU was exceeding WHO guidelines (Table 1). These samples (except Amloki ($6.57x10^4$CFU/g) also exceeded the standard limits of the limit of contamination for herbal medicinal products suggested in European Pharmacopoeia (2007). In a study conducted by Idu *et al*. (2010) mean heterotrophic bacterial count of different medicinal plant samples ranged from 1.3×10^5 CFU/g to 6.7×10^6 CFU/g which is lower than our present study. In another study conducted by Idu *et al*.,(2015) all the polyherbal samples showed high microbial contamination with total bacterial counts ranging from $2.5x10^3$ to $6.4x10^9$ which is also lower than our present study. In another study conducted by Abba *et al*. (2009), the bacterial load ranged from 1.0×10^7 to 1.8×10^8 CFU/g respectively. All these values are lower than the CFU value ($6.57x10^4$ CFU/g to $1.65x10^{15}$ CFU/g) of our present study.

The microbiological load of medicinal plants is the consequence of a number of influences. Medicinal plant products are contaminated by many ways. By their origin, herbal medicines are contaminated by microorganisms from air, water and soil where potentially pathogenic microbes to man may be present and these microbes normally adhered to roots, stems, leaves, flowers and seeds of plants (Adeleye *et al*., 2011). Moreover, these medicines are used in diverse forms and thus have a possibility to carry different microbes originating from soil. Besides, microbial contamination is influenced by a number of environmental factors like humidity, extent of rainfall and temperature during the pre-harvesting and post harvesting periods, the storage conditions and handling practices of processed and crude herbal materials.

Identification of pathogenic bacteria present in the medicinal plant samples was also studied. In this study, *B. cereus* was identified from all the medicinal plant samples taken in this study. Previous studies on medicinal plants have also reported the presence of methicillin and vancomycin resistant highly pathogenic *Bacillus* species (Govender *et al*., 2006; Stickel *et al*., 2009; Kaume *et al*., 2012). *B. cereus* was also isolated and identified from chamomile and other herbs by Martins *et al*. (2001). Study conducted by Idu *et al*. (2010), showed that among the microbial species isolated, *Bacillus* spp. (82.4 %) had the highest prevalence rate among other bacteria. In addition, the presence of *B. cereus*, and *Pseudomonas sp.* like pathogenic bacteria was also reported in the plant samples analyzed by Alwakeel (2008) and Idu (2011).

The occurrence of elevated number of spore forming bacteria is explained by the fact that these bacteria (e.g. *Bacillus* sp.) can produce spores that are resistant to elevated heat, harsh processing and dry conditions. Therefore, they survive in a dormant state on the product for a long time (Famewo *et al*., 2016). Contaminated processing equipment, improper handling of products and inadequate heat processing may help in the survival of these *Bacillus* and *Pseudomonas* species (Frazier and Westhoff, 2003).

Both *Bacillus cereus* and *Pseudomonas gessardii* have pathogenic property and cause different disease to human. *Bacillus cereus* is an emerging food-borne pathogen to human. Several reports have found *B. cereus* to

be associated with acute systemic and local human infections like pneumonia, endophthalmitis, bloody diarrhoea, lung infections, meningitis and gastroenteritis posing severe public health problem (Bottone, 2010; Hilliard *et al*., 2003). The pathogenicity of *B. cereus* depends on its ability to persist, colonise and consequently invade the host tissues (Kamar *et al*., 2013). This bacterium can also produce emetic toxins which are responsible of non-gastrointestinal and gastrointestinal infections (Kotiranta *et al*., 2000). On the other hand, *Pseudomonas gessardii* is an opportunistic pathogen and causes disease in persons with weak immune system (Biaggini *et al*., 2015).

Different studies performed on medicinal plant products have revealed the existence of bacterial pathogens with multiple drug resistance (Oluyege and Adelabu, 2010). In the present study, antibiotic sensitivity test of *Bacillus cereus* and *Pseudomonas gessardii* showed resistance of these bacteria against different antibiotics which is compatible with previous study of Yu *et al*. (2020). *B. cereus* produces β-lactamase, so this result is expected (Bottone, 2010). The antibiotic sensitivity pattern of *B. cereus* also suggested that suspected *B. cereus* infections would not be treated with penicillin and broad-spectrum cephalosporins clinically.

5. Conclusions

This study shows the presence and abundance of *Bacillus cereus* and *Pseudomonas gessardii* in medicinal plant samples rendering them unsafe for human consumption. Therefore, with a view to improving the safety and purity of the herbal products, basic hygiene inspection during herbal medicine preparation, physical characteristics like moisture content, pH and temperature standardization and range of microbiological contamination within an acceptable level are required. Drying of medicinal plant parts after harvesting can limit potential growth of micro-organisms. Besides, as underground and wetter plant parts are more susceptible to contamination than the aerial parts, they should be handled carefully to minimize contamination.

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Data availability

All relevant data are within the manuscript.

Conflict of interest

None to declare.

Authors' contribution

S. M. Rokon-Ud-Doula: methodology, data collection, analysis and manuscript writing; Nazia Afrin: conceptualization, supervision, manuscript writing, reviewing and editing; Md. Maniruzzaman Sikder: data analysis, reviewing and editing; Rasheda Yasmin Shilpi: conceptualization, supervision, reviewing and editing. All authors have read and approved the final manuscript.

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