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Research Article

Estimation of Microbial Propagation and *In-Vitro* Antibacterial Traits of the Commonly Available Plant Exact through Extraction Methods and MIC

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Received: July 30, 2019; **Published:** February 26, 2020

Abstract

Due to the less side effects and potential antimicrobial activity of some plant, flower and roots, scientists are more interested to formulate the new drug from these components. Present study portrayed a complete microbiological profile and anti-bacterial properties of 5 categories of 25 (n = 25) medicinal plants including *Moringa oleifera, Lablab niger, Swertia chirayita, Azadirachta indica* and *Ocimum tenuiflorum* through conventional culture methods, biochemical tests, methanol and ethanol extraction method and minimum inhibitory concentration (MIC) test. The heterotrophic bacterial and fungal contamination was quantified up to 10^6 cfu/g in all the samples. Exploration of pathogens was estimated within the range of 10^3 to 10^6 cfu/g of which *Pseudomonas* spp. was found in *Lablab niger, Ocimum tenuiflorum* and *Moringa oleifera* ($\sim 10^6$ cfu/g) whereas Staphylococcal contamination was evident in almost all samples up to 10^6 cfu/g. *Escherichia coli* was found only in *Moringa oleifera*. Study of antibiogram revealed that *E. coli* was 100% resistant against almost all the antibiotics. *Pseudomonas* spp. and *Staphylococcus* spp. was found to be highly resistant against more than one drugs only Gentamycin and Azithromycin was effective against the 3 bacteria. The *in vitro* antimicrobial activities of the plant extracts were notable against most of the test bacteria. Both ethanolic and methanolic extracts showed anti-bacterial activity against most of the bacteria up to 21 mm as highest and 10 mm as lowest zone diameter. This study also revealed the minimal inhibitory concentration (MIC) of all the samples, which trimmed down the growth of all the tested bacteria. The reflection of drug-resistant pathogens in the plant tested in our study is suggestive of implementing more hygienic manufacturing and processing systems. However, the anti-bacterial traits of the samples revealed the potency of plant against bacteria as well as the biological mechanism of action of such natural medicines.

Keywords: *Medicinal Plant; Microbiological Analysis; Drug Resistant; Antimicrobial Activity; Extraction Methods*

Introduction

Medicinal plants are the prospective sources of antimicrobial agents in different countries [1-3]. Herbal medicines have been used traditionally as drug for their implication in mitigating a number of clinical complications [4-8]. About 60 to 80% of populations in the developing countries use plant derived medicine. According to WHO estimation, 21,000 plants have been found globally to be used for medicinal purposes [9]. Bangladesh is a land of plenty of herbs and plants but the microbial aspect or medicinal usage of these plants have not been well chalked out [10]. In recent years, an important shortfall of using antibiotics has appeared through the emergence of drug resistant bacteria which in turn resulted in the ineffectiveness of antibiotics [11-13]. Moreover, a huge number of reports revealed the

adverse effects and drug interactions resulting in fatal toxicity [14]. Besides, the treatment options of first generation, second generation and even in some particular cases third generation antibiotic becoming limited due to the development of bacterial drug-resistance and the drug-unavailability as a result physicians employing antibiotics appear to be eventually more toxic to the users. Patients with drug-resistant infections are often much more likely to expire and even the survivors often need a longer hospital stays with prolonged healing. Earlier notation of anti-bacterial traits of natural products thus made an array of herbs to be the suitable alternative medication means of antibiotics [15]. Afterwards a number of reports revealed the antagonistic feature of herbs against a wide range of microorganisms as well as proved the lesser side effects with small or no toxicity of the herbal medicines compared to that of antibiotics [16-18]. In addition to the advantages over antibiotics, an important aspect is to be considered that herbs are more likely to harbor a huge number of bacteria including *Escherichia coli, Salmonella* spp., *Shigella* spp., aerobic spores, and the fungal population which usually originating in plantation soil or may be disseminated from organic fertilizer [19,20]. Besides the method of harvesting herbs or herbal products, the production process, unhygienic handling, transportation and inappropriate storage may also cause microbial contamination as well as the chemical toxicity of the herbal medicines [21]. In Bangladesh, huge pharmacological evaluation and ethno-medicinal survey of medicinal plants have been conducted however, the microbiological survey of herbal medicines have not been accomplished well [22,23]. While the research on microbiological assessment of the pharmaceutical products is abundant in this country, such work on the herbal products is quite petite [24,25]. Along these lines, present study portrayed a complete microbiological profile of commonly available medicinal plants including *Ocimum tenuiflorum* (Tulsi), *Azadirachta indica* (Neem), *Lablab niger* (Sheem), *Swertia chirayita* (Chirata) and *Moringa oleifera* (sajna).

Materials and Methods

Study area, sampling, sample processing and microbiological analysis

Five categories of 25 (n = 5) medicinal plants including *Ocimum tenuiflorum* (Tulsi), *Azadirachta indica* (Neem), *Lablab niger* (Sheem), *Swertia chirayita* (Chirata) and *Moringa oleifera* (sajna) were randomly collected during January 2019 to April 2019 following standard protocol [26]. Samples were quickly transported in to the laboratory, and prior to microbiological assay. 10g of each sample was mixed with 90 ml of buffer peptone water (pH 7.2 ± 0.2) in 9:1 ratio and serially diluted up to 10^{-5}.

From the dilution 10^{-4} each of the samples (0.1) ml was introduced on to the nutrient agar and Sabouraud dextrose agar for the isolation of total viable bacteria and fungi, respectively. Subsequently, MacConkey agar, Membrane Fecal Coliform agar (M-FC), Mannitol Salt agar, Cetrimide agar and *Actinomycetes* agar were used as selective media for the quantification of coliforms, fecal coliforms, *Staphylococcus* spp. *Pseudomonas* spp. and *Actinomycetes*, consecutively [27]. All the inoculated plates were incubated at 37°C for 24 hours except SDA plates, which were incubated at 25°C for 48 hours.

Antibiotic susceptibility test

Antibiotic susceptibility traits of the pathogenic isolates were examined (either drug resistant or sensitive) by the disc diffusion assay on Mueller-Hinton agar (MHA, Difco, Detroit, MI) against the commonly used antibiotics following the standard protocol [28,29]. Antibiotic discs such as Penicillin G (10 µg), Gentamicin (10 µg), Oxacillin (1 µg) Amoxicillin (30 µg), Imipenem (30 µg), Erythromycin (15 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg),Trimethoprim-sulfamethoxazole (25 µg), Azithromycin (15 µg), Nalidixic acid (30 µg) and Ampicillin (10 µg) were aseptically placed over the surface of Mueller-Hinton agar plates at spatial distance of 5 mm which was previously lawned with the pathogenic suspensions with standard turbidity (compared to that of the McFarland standard of 0.5).

Antibacterial activities of different medicinal plant extract through agar well diffusion methods

The Mueller-Hinton agar (MHA) plates were prepared followed by Modified agar well diffusion method [30,31]. Lawns of bacterial suspensions including *Escherichia coli, Pseudomonas* spp., *Listeria* spp., *Vibrio* spp., *Klebsiella* spp., *Staphylococcus aureus, Bacillus* spp. (turbidity compared with the McFarland standard) were prepared, wells (8 mm^3) were made, and 100 µl of the direct plant extract were introduced in the well. Normal saline was used as negative controls while the antibiotic discs of gentamicin (10 µg) were used as positive control. All the plates were incubated at 37°C for 12 - 18 hours and examined for formation of the zone of inhibitions (mm).

Solvent extraction

The dried parts of each plant were first ground, and the fine powders were added to 120 ml of ethanol and methanol in Durham's bottle which were kept in shaking water bath at 130 rpm for 24h at 20°C. Extracts were then concentrated in a rotary evaporator under reduced pressure. The dried residual extracts were dissolved in 10% dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/ml [32]. Samples were stored overnight at -20°C until use.

Assay of antimicrobial activity of the plant extract (ethanol and methanol) against laboratory strain

100 µl of the crude extract, ethanol- and methanol extracts at a concentration of ~11.1 mg/ml each were introduced into the wells (8 mm^3) on Mueller-Hinton agar (MHA) plates those were previously lawn with different bacterial culture (*Escherichia coli, Pseudomonas* spp., *Listeria* spp., *Vibrio* spp., *Klebsiella* spp., *Staphylococcus aureus, Bacillus* spp.). Absolute ethanol, methanol and dimethyl sulfoxide (10%) were used as negative controls while the antibiotic discs of gentamicin (10 µg) were used as positive control. Plates were incubated at 37°C for 12 - 18 hours and examined for formation of the zone of inhibitions (mm).

Determination of minimal inhibitory concentration (MIC)

The MIC assay was performed to determine the lowest concentration of herbal medicine which can trim down the extent of viability of the test bacteria [33]. According to the suggested method by Clinical and Standard Laboratory Institute: two fold serial broth dilution method was used to determine the MIC [34]. Overnight culture of each test organisms 100 µl was inoculated into the sterile tube containing Mueller Hinton (MH) broth (Oxoid Ltd, England) at the turbidity adjusted with 0.5 McFarland standard and the different concentration of herbal medicine (32 µL, 64 µL, 128 µL, 256 µL, 512 µL, 1024 µL and 2048 µL) were subjected to introduced into the inoculum containing broth. All the tested tubes were incubated at 37°C for 24 hours. The smallest amount of samples which could retard the multiplication of the tested bacteria as indicated by measuring the zero density, was regarded as MIC.

Results and Discussion

Previously one of our research group unveiled the anti-bacterial traits of *H. rosasinensis, I. coccinea, I. digitata* and *A. cathartic* extracts as well as different herbal medicine which is in consistent to several other studies [35,35]. Several plants are widely known to combat against a range of disease complications; from this point of view, we further turned our attention to investigate the antibacterial potentiality of more 5 different medicinal plants very available in Bangladesh. One of the important aspect of the study was the inhabitant microflora in plants and herbs, not so much studies have been reported so far. Our study showed that almost all samples exhibited huge gathering of spoiling bacterial and fungal flora (Table 1).

Samples	*TVB*[a] (cfu/g)	*Fungi* (cfu/g)	*E. coli* (cfu/g)	*Staphylococcus* spp. (cfu/g)	*Pseudomonas* spp. (cfu/g)
Moringa oleifera (n = 5)	3.2×10^6	3.4×10^6	2.2×10^6	1.5×10^6	0
Lablab niger (n = 5)	6.8×10^6	2.5×10^6	0	2.4×10^5	2.15×10^6
Swertia chirayita (n = 5)	3.6×10^5	3.6×10^6	0	3.6×10^6	4.08×10^3
Azadirachta indica (n = 5)	5.4×10^6	4.5×10^6	0	2.0×10^5	0
Ocimum tenuiflorum (n = 5)	4.8×10^5	2.2×10^5	0	3.2×10^6	4.16×10^5

Table 1: *Microbiological profile of some medicinal plant.*

[a] *Total viable bacteria. The average microbial load has been shown in the table. Fecal coliform, Bacillus spp. and Klebsiella spp. were completely absent in all samples. The experiment has been done in triplicate and the result was reproducible.*

The heterotrophic bacterial and fungal contamination was quantified up to 10^6 cfu/g in all the samples. Exploration of specific pathogenic bacteria was estimated within the range of 10^3 to 10^6 cfu/g of which *Pseudomonas* spp. was found in *Lablab niger* (Sheem), *Ocimum tenuiflorum* and *Moringa oleifera* (Sajna) (~10^6 cfu/g) whereas, Staphylococcal contamination was evident in almost all samples up to 10^6 cfu/g. *Escherichia coli* was found only in *Moringa oleifera* (Sajna).

Drug-resistance trait of the pathogens isolated from medicinal plant samples

The surfacing of drug-resistant bacteria within an array of products (including water, food and pharmaceuticals) resulted in the incompetence of the synthetic drugs or antibiotics [11,12,28,31]. *E. coli* was 100% resistant against almost all the antibiotics. *Pseudomonas* spp. and *Staphylococcus* spp. was found to be highly resistant against more than one drugs only Gentamycin and Azithromycin was effective against the 3 bacteria (Figure 1).

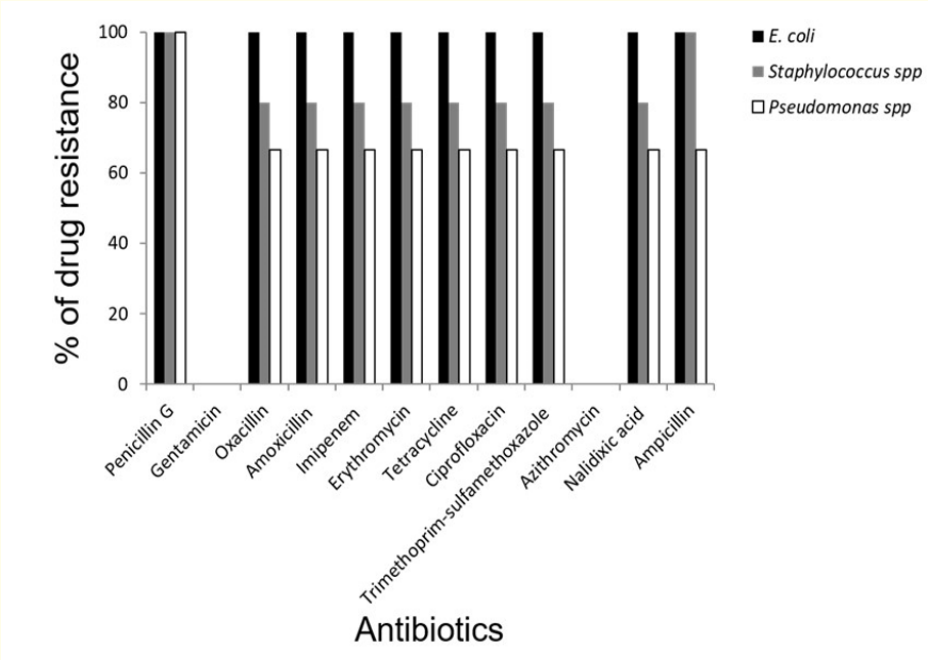

Figure 1: *Resistant phenomenon of the isolates against commonly used antibiotics.*

In-vitro Anti-bacterial activity of different plant extracts

Several studies revealed different herbs and flower extracts to be inhibitory of growth of certain pathogens including *E. coli, P. aeruginosa, S. aureus, B. subtilis, S. pneumoniae* and *Enterobacter aerogenes* [36,37]. According to our study, the direct extract of different plant like *Moringa oleifera* (Sajna), *Lablab niger* (Sheem), *Swertia chirayita* (chirota), *Azadirachta indica* (Neem) and *Ocimum tenuiflorum* (Tulsi) were found to exhibit the bactericidal effects against *Escherichia coli, Pseudomonas* spp., *Listeria* spp., *Klebsiella* spp., *Staphylococcus* spp., *Bacillus* spp. up to 15 mm zone diameter. Only there was no antibacterial effect against *Vibrio* spp. *Moringa oleifera* (Sajna), *Lablab niger* (Sheem) and *Azadirachta indica* (Neem) extracts had no effect against *Pseudomonas* spp. while the growth of *Staphylococcus* spp. was not inhibited by *Moringa oleifera* (Sajna) and *Ocimum tenuiflorum* (Tulsi). *Swertia chirayita* (chirota) and *Ocimum tenuiflorum* (Tulsi) were found to be ineffective against *Listeria* spp. The crude fraction and the residual extracts also showed no activity (Table 2).

Test bacteria	Bacteria								
	E. coli	***Pseudomonas* spp.**	***Vibrio* spp.**	***Bacillus* spp.**	***Klebsiella* spp.**	***Staphylococcus* spp.**	***Listeria* spp.**	***Negative control* (BPW)**	***Positive control* (Gentamicin 10 µg)**
	Zone diameter in (mm)								
Moringa oleifera	11	0	0	14	11	0	11	0	18 mm
Lablab niger	14	0	0	15	13	12	13	0	22 mm
Swertia chirayita	15	12	0	15	13	0	0	0	16 mm
Azadirachta indica	13	0	0	11	14	14	15	0	22 mm
Ocimum tenuiflorum	16	15	0	12	11	0	0	0	18 mm

Table 2: *Antimicrobial activity of five medicinal plant extracts.*

The ethanolic and methanolic extracts of *Moringa oleifera* was found to form zone of inhibition against both Gram negative and Gram positive bacteria. The ethanolic extract showed 21, 14, 11, 12, 18, 10 mm zone of inhibition against *Pseudomonas* spp. *Vibrio* spp. *Bacillus* spp. *Klebsiella* spp. *Staphylococcus* spp. and *Listeria* spp. consecutively while the methanolic extract showed bactericidal activity against *Vibrio* spp. and *Staphylococcus* spp. up to 17 mm zone diameter. However, no activity was scored against *E. coli* and *Salmonella* spp. (Table 3).

Test bacteria	Zone of Inhibition in diameter (mm)						
	Crude fraction	**Negative control (BPW)**	**Negative control (Ethanol)**	**Ethanol extract**	**Negative control (Methanol)**	**Methanol extract**	**Positive control (Gentamicin 10 µg)**
E. coli	0	0	8	0	0	0	16.80
Pseudomonas spp.	0	0	0	21	0	0	28.44
Vibrio spp.	0	0	0	14	0	14	18.01
Bacillus spp.	0	0	7	11	0	0	22.00
Klebsiella spp.	0	0	0	12	0	0	18.83
Staphylococcus spp.	0	0	0	18	0	17	22.00
Listeria spp.	0	0	0	10	0	0	23.00
Salmonella spp.	0	0	0	0	0	0	29.22

Table 3: *Antimicrobial activity of Moringa oleifera extracts.*

In case of *Lablab niger* ethanol extract, the activity was found on *E. coli* (11 mm), *Klebsiella* spp. (16 mm) and *Bacillus* spp. (18 mm) whereas no antibacterial activity was coined against others (Table 3). On the other hand, the methanol extract showed zone of inhibition 14, 10, 13 mm against *E. coli, Pseudomonas* spp. and *Listeria* spp. consecutively (Table 4).

The extracts of *Swertia chirayita* was found to be effective against *E. coli* (11 mm), *Pseudomonas* spp. (12 mm), *Bacillus* spp. (11 mm), *Klebsiella* spp. (11 mm), *Staphylococcus* spp. (11 mm) and *Salmonella* spp. (15 mm). While *Vibrio* spp. and *Listeria* spp. were found to be resistant. No antibacterial activity was found in case of methanolic extract (Table 5).

Test bacteria	Zone of Inhibition in diameter (mm)						
	Crude fraction	Negative control (BPW)	Negative control (Ethanol)	Ethanol extract	Negative control (Methanol)	Methanol extract	Positive control (Gentamicin 10 µg)
E. coli	0	0	8	11	0	14	16.80
Pseudomonas spp.	0	0	0	0	0	10	28.44
Vibrio spp.	0	0	0	0	0	0	18.01
Bacillus spp.	0	0	7	18	0	0	22.00
Klebsiella spp.	0	0	0	16	0	0	18.83
Staphylococcus spp.	0	0	0	0	0	0	22.00
Listeria spp.	0	0	0	0	0	13	23.00
Salmonella spp.	0	0	0	0	0	0	29.22

Table 4: *Antimicrobial activity of Lablab niger extracts.*

Test bacteria	Zone of Inhibition in diameter (mm)						
	Crude fraction	Negative control (BPW)	Negative control (Ethanol)	Ethanol extract	Negative control (Methanol)	Methanol extract	Positive control (Gentamicin 10 µg)
E. coli	0	0	8	11	0	0	16.80
Pseudomonas spp.	0	0	0	12	0	0	28.44
Vibrio spp.	0	0	0	0	0	0	18.01
Bacillus spp.	0	0	7	11	0	0	22.00
Klebsiella spp.	0	0	0	11	0	0	18.83
Staphylococcus spp.	0	0	0	11	0	0	22.00
Listeria spp.	0	0	0	0	0	0	23.00
Salmonella spp.	0	0	0	15	0	0	29.22

Table 5: *Antimicrobial activity of Swertia chirayita extracts.*

In case of *Azadirachta indica*, the both extraction (ethanol and methanol) showed antibacterial activity against all the tested bacteria. The maximum zone of inhibition was recorded 18 mm against *Pseudomonas* spp. in case of ethanolic extract while the zone diameter 19 mm was recorded against *Salmonella* spp. for methanolic extraction (Table 6).

The extraction (ethanol and methanol) of *Ocimum tenuiflorum* found to be active against almost all the tested bacteria excluding *Salmonella* spp., and *Listeria* spp. In case of *Ocimum tenuiflorum*, the utmost zone was calculated 15 mm for both extraction (Table 7).

From our findings, it is evident that the *in vitro* antimicrobial activities of the plant extracts, especially after ethanol and methanol treatment, were prominent against microorganisms. Also, interestingly the ethanol extract of all the samples exhibited antimicrobial activity against *Bacillus* spp. The earlier studies unveiled the presence of antimicrobial activity in the solvent extracts of the flower samples could possess the potential of being source of commercially dispensable herbal antimicrobial agents against different topical and enteric diseases [38,39].

Test bacteria	Zone of Inhibition in diameter (mm)						
	Crude fraction	Negative control (BPW)	Negative control (Ethanol)	Ethanol extract	Negative control (Methanol)	Methanol extract	Positive control (Gentamicin 10 µg)
E. coli	0	0	11	17	0	12	16.80
Pseudomonas spp.	0	0	0	18	0	15	28.44
Vibrio spp.	0	0	0	16	0	11	18.01
Bacillus spp.	0	0	11	11	0	13	22.00
Klebsiella spp.	0	0	0	12	0	12	18.83
Staphylococcus spp.	0	0	0	15	0	11	22.00
Listeria spp.	0	0	0	17	0	15	23.00
Salmonella spp.	0	0	0	16	0	19	29.22

Table 6: *Antimicrobial activity of Azadirachta indica extracts.*

Test bacteria	Zone of Inhibition in diameter (mm)						
	Crude fraction	Negative control (BPW)	Negative control (Ethanol)	Ethanol extract	Negative control (Methanol)	Methanol extract	Positive control (Gentamicin 10 µg)
E. coli	0	0	8	15	0	14	15
Pseudomonas spp.	0	0	0	15	0	14	14
Vibrio spp.	0	0	0	15	0	15	18
Bacillus spp.	0	0	7	11	0	11	22
Klebsiella spp.	0	0	0	11	0	14	18.83
Staphylococcus spp.	0	0	0	11	0	11	22
Listeria spp.	0	0	0	0	0	0	15
Salmonella spp.	0	0	0	0	0	0	16

Table 7: *Antimicrobial activity of Ocimum tenuiflorum extracts.*

Determination of MIC of the plant extract

In addition, the *in vitro* anti-bacterial activity of the samples was further supported by observing the result of MIC (Table 8). In this parameter all the samples were found to show their anti-bacterial activity against different tested bacteria; i.e. *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., *Bacillus* spp., *Salmonella* spp., *Listeria* spp., *Staphylococcus* spp., and *Vibrio* spp. The most effective MIC of the sample was scored at 128 µL while the high value was noted to be 1024 µL (Table 8). For example sample 1, the ideal MIC was found to be 512 µL against all the tested pathogenic bacteria except *Bacillus* spp. which was found to be inhibited at the concentration of 1024 µL. Sample 2 and 3 revealed the MIC at a maximal level of 256 µL to stall the bacterial growth. For sample 4, the MIC was recorded to be not more than 512 µL to inhibit any of the tested bacterial growth. Sample 5 showed the MIC potential at 128 µL against most of the tested pathogenic bacteria while 512 µL and 1024 µL were found to be effective against *Bacillus* spp. and *Staphylococcus* spp., respectively. (Table 8).

The experiments were conducted three times independently, and the results were found to be reproducible. One representative data has been shown.

Sample	Organisms							
	E. coli	***Klebsiella* spp.**	***Pseudomonas* spp.**	***Bacillus* spp.**	***Salmonella* spp.**	***Listeria* spp.**	***Staphylococcus* spp.**	***Vibrio* spp.**
Moringa oleifera	512 µL	512 µL	512 µL	1024 µL	512 µL	512 µL	512 µL	512 µL
Lablab niger	256 µL	256 µL	256 µL	1024 µL	256 µL	512 µL	256 µL	256 µL
Swertia chirayita	256 µL	256 µL	128 µL	256 µL	256 µL	256 µL	256 µL	256 µL
Azadirachta indica	512 µL	512 µL	512 µL	1024 µL	512 µL	512 µL	256 µL	512 µL
Ocimum tenuiflorum	128 µL	128 µL	128 µL	512 µL	128 µL	128 µL	1024 µL	128 µL

Table 8: *Minimum Inhibitory Concentration (MIC) of the sample.*

However, agar well diffusion test may not be a suitable one to determine the antibacterial activity of natural compounds. The rate of diffusion of natural antimicrobials can be strongly affected by the polarity, the concentration, the molecular size, etc. of the compounds. This fact was evident from our study findings. When the results of agar well diffusion method and MIC assay were compared, a clear discrepancy was apparent for some samples.

Conclusion

A large number of studies have been carried out so far to detect the microbiological contamination level in flowers and others herbs; nevertheless, the sustainable capacity of such contamination is still now difficult to understand. Our study revealed a complete profile of microbial contamination in flower and measured the anti-bacterial activity of their ethanol and methanol extracts. The findings may largely assist to design a model for the development of new herbal medicines thereby augmenting the public health safety.

Acknowledgements

We thank Microbiology Laboratory, Stamford University Bangladesh for laboratory facilities.

Conflict of Interest

The authors have declared no conflict of interest.

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