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Assessment of microbiological contamination and the *in vitro* demonstration of the anti-bacterial traits of the commonly available local fruit blend within Dhaka Metropolis

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ABSTRACT
Present study assessed the microbial load 5 categories of local fruits commonly consumed within the Dhaka metropolis community. All samples were found to be populated with a huge number of bacteria (~10^9 cfu/g). *Staphylococcus* spp. was the most prevalent, especially in the orange skin; whereas *Pseudomonas* spp. was detected in the apple and dragon fruit skins, and within both the skin and flesh portions of guava samples. *Listeria* spp. was detected in all samples either in the skin or in the flesh portions but not in both. A nominal fungal growth was observed in the samples except orange sample. Significant anti-bacterial activity was noticed for malta and guava samples, especially within the flesh portions of guava with the most potential activity against *Pseudomonas* spp. and *Vibrio* spp. Apple and orange blends exhibited moderate activity, and the weakest activity was measured for the dragon fruit blends.

Keywords: Microorganisms; contamination; Guava (*Psidium guajava)*; malta (*Citrus sinensis)*; apple (*Malus domestica)*; orange (*Citrus reticulate)*; dragon fruit (*Hylocereus polyrhizus*); public health safety.

1. Introduction
Fresh fruits are an excellent source of essential nutrients, and this, along with other health benefits, has made it a major food source globally [1]. Although the consumption of fresh fruit and vegetables remain popular worldwide, conversely the elevation in the microbial pathogenic proliferation continues to be publicly alarming [2-4]. In developing countries microbial contamination regularly occurs in the plantation fields, due to the contaminated irrigation water along with unhygienic handling especially during harvesting [5]. Such fresh food borne infections especially caused by *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Aeromonas* spp., *Staphylococcus* spp., *Streptococcus* spp., *Vibrio* spp. and *Pseudomonas* spp., have long been known to contribute to the onset of an array of diseases leading to fatality in both developed and developing nations [3, 6-8].
Another alarming issue stems from the growing increase in anti-bacterial resistance, shifting the focus to the alternative forms of natural products with anti-bacterial traits [6-9]. Ease of availability of over the counter antibiotics and the unregulated use and abuse of antibiotics have greatly contributed to the rise in the drug resistance [10-13]. The enhanced nutritional advantage of a number of fruits escalated their possible applications for medications after required processing which indeed have globally been conveyed in quest of the synchronized regulation of public health safety besides applications of traditional antibiotics to combat against pathogenic microorganisms [14-21].
Regarding the activity of fruits against pathogens, Interestingly, earlier we noticed significant anti-bacterial traits of the methanol and ethanol extracts of both the local and imported fruits; i.e., guava (*Psidium guajava)*; malta (*Citrus sinensis)*; apple (*Malus domestica)*; orange (*Citrus reticulate)*; dragon fruit (*Hylocereus polyrhizus*) samples [22], which further led us to examine the anti-bacterial activity of the local samples that are more readily available than the imported ones, without treating or extracting with methanol and ethanol solvents. Along these lines, our present study first assessed the microbiological quality of these local fruits available within Dhaka metropolis, and further chalked out the anti-bacterial activity of the local samples.

2. Materials and methods

2.1. Sampling and sample processing

Five categories of local fruits including Guava (*Psidium guajava)*, malta (*Citrus sinensis)*, apple (*Malus domestica)*, orange (*Citrus reticulate)* and dragon fruit (*Hylocereus polyrhizus*) samples were randomly collected from street vendors or local groceries within the city of Dhaka, Bangladesh. Samples were collected early in the morning and transported quickly to the laboratory and immediately processed for further tests [22, 23].

2.2. Microbiological analysis and confirmative biochemical identification

Identification and enumeration of microorganisms were carried out according to standard procedures as described earlier [2, 3]. Twenty five (25) grams of vegetable samples were homogenized with 225 ml of normal saline (Crystal sodium chloride) and diluted up to 10^{-6}, followed by plating in different selective and differential culture media using the standard methods [24]. For the quantification of total viable bacteria (TVB) and fungi, 0.1 ml of each sample was introduced onto the Nutrient agar and Sabouraud's dextrose agar (Oxoid Ltd., Basingstoke, Hampshire, England) plates, respectively, by means of spread plate technique [24]. Plates were incubated at 37 °C for 24 hours and at 25 °C for 48 hours for total viable bacteria and fungi, respectively.

2.3. Estimation of specific bacterial pathogens

From the dilutions 10^{-3} and 10^{-5}, 0.1 ml of each sample was spread onto the Membrane fecal coliform (MFC) agar and MacConkey agar (Oxoid Ltd., Basingstoke, Hampshire, England) for the enumeration of total fecal coliform (TFC), and coliforms (*Escherichia coli* and *Klebsiella* spp.), followed by incubation at 44.5 °C and 37 °C for fecal coliform and coliforms, respectively for 24 hours. Likewise, *Staphylococcus* spp. was isolated by adding 0.1 ml of diluted sample onto the Mannitol salt agar (MSA) (Oxoid Ltd., Basingstoke, Hampshire, England) following incubation at 37 °C for 24 hours.

2.4. Enrichment methods

After homogenization of 25 grams of vegetable samples with 225 ml of normal saline, 10 ml of samples were transferred into 90 ml of selenite cysteine broth and alkaline peptone water (Oxoid Ltd., Basingstoke, Hampshire, England) for the enrichment of *Salmonella, Shigella*, and *vibrio* spp., respectively and incubated at 37 °C for 4-6 hours [3]. Samples were then diluted up to 10^{-6} and 0.1 ml of samples from each of the 10^{-3} and 10^{-5} dilutions were spread onto *Salmonella-Shigella* agar and thiosulfate citrate bile salt sucrose agar (Oxoid Ltd., Basingstoke, Hampshire, England) for the isolation of *Salmonella* spp. *and Shigella* spp., *and Vibrio* spp., respectively. Plates were incubated at 37 °C and the appearance of typical colonies (if any) was noticed within for 24-48 hours. Finally, the identity of all the isolates was confirmed by conducting a series of biochemical tests as described previously [2, 3, 24, 25].

2.5. Determination of Antimicrobial Activity

Antimicrobial activity was determined by using the agar well diffusion methods as previously described [6, 22, 26]. Suspensions of 9 test bacteria (*Bacillus* spp., *Pseudomonas* spp., *Vibrio* spp., *Escherichia coli*, *Klebsiella* spp., *Staphylococcus* spp., *Listeria* spp., *Salmonella* spp., and *Aeromonas* spp.) were prepared in normal saline resulting an average bacterial load of 10^6 cfu/ml (compared to the 0.5 mL McFarland standard). Each of the test bacterial suspensions was separately spread evenly over the separate Muller- Hinton agar (Oxoid Ltd., Basingstoke, Hampshire, England) to prepare the uniform lawns. Wells (8 mm^3) spanning through the Muller- Hinton agar was generated by using cork-borer. Each of the fruit blend samples; i.e., skin and flesh portions (100 µl each) was then introduced separately in the specified well along with the disc of gentamicin 10 µg (Oxoid Ltd., Basingstoke, Hampshire, England) as the positive control and 100 µl normal saline as the negative control [8]. Sterile cork borers were used to create 8 mm wells, after plate had dried. 100 µl of each fruit blend was placed into separate wells, dried and then incubated at 37 °C overnight. Test organisms normal saline and gentamicin 10 µg were used as positive and negative control, respectively. Presence of clear zone (if any) around the samples applied was analytical for the existence of the anti-bacterial traits of the samples studied.

3. Results and Discussion

3.1. Prevalence of bacteria and fungi in the local fruit samples

Fresh fruits are known to impart essential health benefits while conversely may also serve as the potential source of microbial dissemination [2-4]. In our study, almost all the fruit samples were found to be contaminated with a huge number of bacteria, being the most prevalent within the skin portions of the guava samples (10^9 cfu/g); while the flesh portion of this fruit were found to be populated with a bacterial population of 10^6 cfu/g. (Table 1).

Table 1: Isolation and enumeration of microorganisms in local fruits

Fruit samples	Sample portions	TVB* (cfu/g)	Fungi (cfu/g)	*Escherichia coli* (cfu/g)	*Salmonella* spp. (cfu/g)	*Staphylococcus* spp. (cfu/g)	*Pseudomonas* spp. (cfu/g)	*Listeria* spp. (cfu/g)	*Vibrio* spp. (cfu/g)
Guava	Skin	2.2×10^9	2.5×10^2	1.8×10^2	3.0×10^2	1.0×10^2	1.1×10^3	3.5×10^1	3.0×10^2
	Flesh	1.8×10^6	1.2×10^2	0	4.0×10^1	2×10^3	3.5×10^3	0	0
Apple	Skin	3.8×10^7	2.2×10^2	3.0×10^2	4.0×10^1	4.0×10^3	5.0×10^3	2.0×10^1	0
	Flesh	2.5×10^6	3.5×10^2	1.5×10^2	2.0×10^1	2.8×10^2	0	0	0
Orange	Skin	3.6×10^6	0	0	0	2.6×10^5	0	0	0
	Flesh	2.3×10^5	0	0	0	9.0×10^3	0	4.0×10^2	0
Malta	Skin	4.6×10^4	2.1×10^2	0	0	1.9×10^2	0	0	0
	Flesh	6.0×10^3	1.3×10^2	0	0	5.0×10^2	0	7.0×10^1	0
Dragon Fruit	Skin	4.5×10^7	3.5×10^2	5.5×10^2	0	3.7×10^3	4.3×10^2	0	0
	Flesh	2.6×10^7	2.8×10^2	1.0×10^2	0	2.1×10^2	0	1.5×10^2	0

* TVB = Total viable bacteria

Fecal coliform and *Shigella* spp. were completely absent from all sample. *Vibrio* spp. was present only in guava samples. The experiment has been done in triplicate and the result was reproducible.

Compared to the relatively low number of bacteria in malta samples (10^3–10^4 cfu/g), the samples studied from apple, orange and dragon fruits were found to harbor bacteria within a range of 10^5–10^7cfu/g. Except for the orange samples, growth and proliferation of fungi (~10^2 cfu/g) were observed in the other 4 samples within both the skin and flesh portions (Table 1). The fruit samples are assumed to get contaminated from the plantation soils, irrigation waters, from manures, or even from animal wastes during cultivation, transportation and further processing [27]. Besides, microorganisms are known to gain access more frequently to the fresh fruit surface due to unhygienic handling and washing with contaminated water during storage [27, 28, 29].

3.2. Existence of pathogenic bacteria among the samples studied

As manifested from our earlier works, a number of enteric complications are triggered by the food borne microbial pathogens [2, 3, 30]. Current study also delivered the similar scenario since a huge number of bacterial pathogens and fungi have been demonstrated in the fruit samples tested. In the current study, among the specific pathogens identified within the fruit samples, staphylococcal burden was observed in all samples; while the bacterial prevalence was most prominent in the orange skin samples (~10^5 cfu/g) (Tables 1 and 2). Growth of *Pseudomonas* spp. was noticed in the skin portions of apples and dragon fruit samples while the guava samples were found to be populated by this bacterium within both the skin and flesh portions. *Listeria* spp. was detected in all samples either in skin or in the flesh portions. Among the enteric bacteria, neither fecal coliform nor *Shigella* spp. was detected in any sample; however *Vibrio* spp. (~10^2 cfu/g) was found to be present only in the guava samples. Presence of *Salmonella* spp. was noticed in the guava and apple samples (10^1 - 10^2 cfu/g), and the other 3 samples were complete devoid of this pathogenic bacterium.

Table 2: Biochemical identification of the pathogenic isolates found in the local fruit samples.

Pathogenic microorganisms	TSI			H_2S reaction	Indole production	MR test	VP test	Citrate utilization	Motility test	Oxidase test
	Slant	Butt	Gas							
E. coli.	Y	Y	+	-	+	+	-	+	+	-
Salmonella spp.	R	Y	-	+	-	+	-	-	+	-
Vibrio spp.	Y	Y	-	-	+	+	-	+	+	+
Staphylococcus spp.	Y	Y	-	-	-	+	+	-	-	-
Listeria spp.	Y	Y	-	-	-	+	+	-	+	-
Pseudomonas spp.	R	R	-	-	-	-	-	+	+	+

TSI = Triple Sugar Iron Test, Y = Yellow (Acid), R = Red (Alkaline), MR = Methyl red, VP = Voges-Proskauer

3.3. Anti-bacterial Activity of fruit blends

While an array of medicinal plants has so far been reported with their effectiveness in perspective of Bangladesh and some other countries within Asia, the effective strategy to identify fruits for mitigating pathogenic microorganisms has not been has not been endorsed properly [16, 31]. Being led by such limitations on the study of anti-microbial application of fruits, current investigation further emphasized on the analysis of the local fruit samples to investigate their intrinsic anti-bacterial attributes.

3.4. Potential anti-bacterial trait of malta and guava samples

Out of all samples tested, comparatively stronger anti-bacterial activity was noticed in case of malta and guava samples (Table 3). The malta skin blends were observed to be moderately active against *Bacillus* spp., *Staphylococcus* spp., *Aeromonas* spp. and *E. coli* with a zone size of inhibition of 9.5-12 mm; however, the flesh portions exhibited the activity against 7 test bacteria with the highest activity against *Staphylococcus* spp. With a bit discrepancy, no activity was scored against *Aeromonas* spp. while both the skin and flesh portions of malta samples were inactive against *Salmonella* spp. The skin portions of guava exhibited a moderate anti-bacterial activity (with a zone size of inhibition within approximately 9-14 mm) against 5 test bacteria (*Pseudomonas* spp., *E. coli*, *Bacillus* spp., *Vibrio* spp. and *Aeromonas* spp.) while the flesh portions were found to bear most potential anti-bacterial activity against *Pseudomonas* spp. and *Vibrio* spp. (with an inhibition zone of around 20 mm), and a relatively moderate activity against *E. coli* and *Salmonella* spp. (Table 3).

3.5. Moderate anti-bacterial trait of apple and orange samples

Compared to malta and guava sample blends, a relatively weaker anti-bacterial activity was scored in case of apple and orange samples (Table 3). The apple skin portions were noticed to exhibit a moderate anti-bacterial activity against *Pseudomonas* spp. and *Listeria* spp., with a relatively lower activity against *Klebsiella* spp.; while the flesh portions were found to be moderately active against *Pseudomonas* spp. and *Staphylococcus* spp., and a relatively lower activity against *Listeria* spp. and *Aeromonas* spp. The skin portions of orange blends exhibited a moderate or even low anti-bacterial activity (with an inhibition zone of 9-12 mm) against *Listeria* spp., *Bacillus* spp. and *Staphylococcus* spp.; while a moderate activity of the flesh portions was observed against *Pseudomonas* spp., *Staphylococcus* spp. and *Bacillus* spp. (zone of inhibition of 12-13 mm), with a comparatively weak activity against *Listeria* spp.

3.6. Least anti-bacterial trait posed by the dragon fruit blends

The skin portions of dragon fruit blends exhibited the lowest activity among all samples tested (only a weak activity against *E. coli*). In contrast the flesh portions were found to be active against *Staphylococcus* spp. and *Pseudomonas* spp.

Table 3: *In vitro* anti-bacterial activity of the local fruit blends

Local fruit samples	Sample blends	Zone of inhibition (mm) against test bacteria								
		***Bacillus* spp.**	***Pseudomonas* spp.**	***Vibrio* spp.**	***Escherichia coli***	***Klebsiella* spp.**	***Staphylococcus* spp.**	***Listeria* spp.**	***Salmonella* spp.**	***Aeromonas* spp.**
Guava	Skin	11.64	14.39	9.55	12.64	0	0	0	0	9.2
	Flesh	0	20.39	19.13	10.53	0	0	0	9.17	0
Apple	Skin	0	11.81	0	0	7.9	0	11	0	0
	Flesh	0	12.9	0	0	0	12	7.9	0	7.5
Orange	Skin	10	0	0	0	0	9	12	0	0
	Flesh	11.90	13.02	0	0	0	13.4	9.5	0	0
Malta	Skin	12	0	0	9.5	0	11	0	0	10
	Flesh	12.95	12.25	10.4	10.8	9.3	16.9	11.9	0	0
Dragon fruit	Skin	0	0	0	9.7	0	0	0	0	0
	Flesh	0	10	0	0	0	12	0	0	0

3.7. Susceptibility of test bacteria against the fruit blends

Among all test bacteria, the susceptibility order was noticed mostly for *Pseudomonas* spp. (against 7 sample blends), followed by *Staphylococcus* spp. (against 6 samples). *Bacillus* spp., *E. coli* and *Listeria* spp. were found to be susceptible against 5 samples each. *Vibrio* and *Aeromonas* spp. exhibited relatively lower susceptibility (against 3 samples each); whereas *Klebsiella* spp. was susceptible against 2 samples, and *Salmonella* spp. against only one sample (Table 3).

As stated earlier, the unauthorized random practice of antibiotic usage, improper treatment of effluent, industrial and pharmaceutical wastes prior to discard into the environment, and finally the horizontal transfer of drug-resistance genes have been known to result in commencement of disease medication problems specifically in the dense populated developing countries like Bangladesh [11, 13]. In this context, the known active compounds such as anti-oxidants with the anti-bacterial properties of the natural herbs including fruits may be an appropriate complementary approach to minimize the compilations imposed by the irregular use of antibiotics [16-22]. Current investigation, apart from our recent study, has chalked out both the microbiological hazards as well as the antimicrobial attribute of the local fruits [22]. The results presented here are in cohort with the other studies which also revealed the significant anti-bacterial potential of fruits against microbial pathogens [1, 6-9, 32].

4. Conclusion

Overall, in cohort with our recent findings using the alcoholic extracts, the current study also revealed a noteworthy anti-bacterial feature within the local fruit blends without solvent extraction as has been done before. The activity was noticed to be significant especially within the malta and guava samples. Such an *in vitro* demonstration of the anti-bacterial activity of the fruits might be scaled up for the purpose of natural medicine formulation, which ultimately would be capable of a better management of the overall public health. Another new aspect of the present study is projected through the microbiological contamination frequency of the fruits, which in turn, may aid to the important concern on the better hygienic handling and processing of these fruits to maintain the public health safety.

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6. Conflict of interest statement

Authors have declared no conflict of interest.

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