

# Isolation and quantification of bacterial isolates from citrus fruits and determination of their anti-bacterial activity against selected pathogens

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The present study was undertaken with the aim of investigating the isolation and quantification of microorganisms from the citrus fruit samples collected from different areas of Dhaka city. Out of ten samples studied, the range of total viable bacterial proliferation was approximately  $10^2$  to  $10^7$  cfu/g. Among the specific bacterial pathogens, prevalence of *Klebsiella* spp. was found in five samples, *Listeria* spp., *Staphylococcus* spp. and *Pseudomonas* spp. in four samples and *Vibrio* spp. in three samples only. The presumptive identification of these isolates was done by the conventional cultural, microscopic and biochemical tests. Fungal growth was also observed in four samples within the range of  $1.2 \times 10^3$  to  $3.6 \times 10^3$  cfu/g. Interestingly, *Prunus mume/domestica* (plum) showed the anti-bacterial activity against all the laboratory bacterial isolates tested. Among other samples, *Tanarindus bacilus* (tamarind) was found to exhibit activity against *Escherichia coli*, *Pseudomonas* spp., *Salmonella* spp., *Vibrio* spp., and *Listeria* spp. On the other hand, *Monifera indica* (mango) showed anti-bacterial efficacy against *E. coli*, *Staphylococcus* spp. and *Listeria* spp. and *Citrus limon* (lemon) only against *Pseudomonas* spp. and *Listeria* spp.

**Key words:** Citrus fruits; Pathogenic bacteria; fungi; Anti-bacterial activity

The term citrus fruit comprises various types of fruits and its related products, principally oranges, accounting for about 70% of citrus output, and the others including tangerines, mandarins, clementine, satsuma, lemon, lime and grapefruits (1). Because of the fresh-like characteristics and the increasing need of low-caloric food products along with the trait to combat against cardiovascular complications and cancerous transformations, the consumption of citrus fruits and fruit juices has significantly risen over the last few years (2). However, due to inappropriate manipulation during the handling, cutting, shredding, and maintenance of the fruit at ambient temperature and storage conditions, both pathogenic and deteriorative microorganisms may contaminate a product, thus increasing the risk of microbial diseases and spoilage (3). Indeed, the incidence of outbreaks and cases of illness caused by consumption of citrus fruits and unpasteurized juices has increased in the last years (4).

Besides the mass use of antibiotics for the treatment of disease, employment of plant extracts and phytochemicals with the known antimicrobial properties appeared with great impact in the therapeutic treatment process (5-9). Citrus flavonoids are well known to possess a large range of biological activity including anti-bacterial, anti-fungal, anti-diabetic, anti-cancer and

anti-viral activities (1, 10). Flavonoids can act as direct antioxidants and free radical scavengers, and have the capacity to modulate enzymatic activities and inhibit cell proliferation (11). In plants, they seem to play a defensive role against invading pathogens, including bacteria, fungi and viruses (12). Preparation from peel, flowers and leaves of bitter orange (*Citrus aurantium*) are popularly consumed in order to minimize the disorders of central nervous system (13). Citrus fruits have been a matter of interest for the extraction of antimicrobial metabolites by a large number of researchers (14-16). Several studies have shown the antioxidant features of medicinal fruit juices that are rich in phenolic compounds. Natural antioxidants are mainly derived from plants in the form of flavonoid, phenolic acids and tocopherols etc. (17).

Massive use of non-prescribed medicines due to the lack of legislative regulations may trigger the onset of treatment mediated complications related to drug-resistance (18). Such a circumstance could stipulate alternative treatment strategies based on natural medication approaches (19, 20). In Bangladesh a range of medicinal plants have been identified so far; however, such medicinal properties within the citrus fruits have not still been identified (19). Thus, in the present investigation emphasized not only on the bacterial contamination extent within the citrus fruits but also attempted to measure the anti-bacterial property of different citrus fruits commonly available and readily consumed in Bangladesh.

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## MATERIALS AND METHODS

**Study area and sampling.** Samples were collected from different local markets of Dhaka city such as Rampura, Shantinagar, Moghbazarg and Mailbagh area in different time intervals and transported to the laboratory as soon as possible according to the method suggested earlier (21). For the identification and enumeration of pathogenic bacteria and fungi, at first 10g of each sample was taken, then blended with 90 ml normal saline (pH 7.8) & diluted up to  $10^{-5}$  (22).

**Total viable bacterial count (TVBC), total fungal load and total coliform count (TCC).** The enumeration was performed by spreading 0.1 ml sample from the  $10^{-5}$  dilution onto nutrient agar (NA) and 0.1 ml from the  $10^{-3}$  dilution onto Sabouraud Dextrose Agar (SDA) for the determination of total viable bacterial count (TVBC) and total fungal load, respectively (23). Plates were incubated at 37 °C for 24 hours and at 25 °C for 48 hours for TVBC and total fungal load respectively. For the detection of total coliform count (TCC), 0.1 ml sample from the dilution  $10^{-3}$  was spread onto MacConkey agar media. The plates were incubated at 37 °C for 24 hours (24, 25). Finally, several biochemical tests were carried out for the final confirmation of coliforms (22).

**Isolation of *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Staphylococcus* spp. and *Pseudomonas* spp.** An aliquot of 1 ml sample was transferred into each of 9 ml of Selenite Cysteine broth (SCB) and Alkaline Peptone Water (APW) for the enrichment of *Salmonella* spp., *Shigella* spp. and *Vibrio* spp., respectively and incubated at 37 °C for 6 hours. After enrichment, samples were diluted up to  $10^{-4}$  and then 0.1 ml sample from  $10^{-2}$  and  $10^{-3}$  was spread onto Salmonella-Shigella (SS) agar and Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar (23). *Staphylococcus* spp. and *Pseudomonas* spp. were isolated from the Mannitol Salt agar (MSA) and *Pseudomonas* agar respectively. After incubation at 37 °C for 24 hours, the typical colony characteristics on these media were observed (24). For further identification, biochemical traits of these isolates were tested following standard methods (22, 26).

**Determination of anti-bacterial activity of the fruit samples.** The antibacterial activity of the fruit samples was demonstrated by agar well diffusion method (27). Briefly, fruit blends were used directly on the Mueller-Hinton agar (MHA) media. At first, the bacterial pathogens (*Escherichia coli*, *Klebsiella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Salmonella* spp., *Vibrio* spp., and *Listeria* spp.) were introduced evenly over the MHA separately using cotton swab, followed by making hole (8 mm<sup>3</sup>) on the MHA by cork borer. Each of the blends was then introduced with the samples (volume of 100 µl with a concentration of 11 µg/µl) separately in the specified hole with a positive control antibiotic disc- Gentamicin (GEN, 10 µg) and the negative control (normal saline). Presence of clear zone around the sample solution (if any) indicated the presence of antibacterial activity (28, 29).

## RESULTS AND DISCUSSION

Most of the fruits are consumed in their raw states and may lead to the onset of human diseases that may put

the overall public health at a severe risk (30). Microorganisms are generally associated in a number of ways with the different types of fresh foods affecting the overall quality and health hygiene (23, 31). Fruits are contaminated easily with bacterial pathogens by the principle of dissemination of bacterial infection and a single infected citrus fruit can be the root of infection to other fruits during storage and on transport (32). In the present study, the load of *Vibrio* spp., *Salmonella* spp. and *Shigella* spp. were found to be nil before enrichment. However, after enrichment only *Vibrio* spp. was estimated within a range of  $1.8 \times 10^2$  to  $3.1 \times 10^2$  cfu/g (Table 1). Total viable bacterial count (TVBC) in all the samples observed in this study was within the range of  $10^2$  to  $10^7$  cfu/g. Among the specific pathogens, *Klebsiella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Vibrio* spp. and *Listeria* spp. were biochemically identified (Table 2). *Klebsiella* spp. was found to exhibit growth in five out of ten samples (Table 1). Surprisingly, both *Pseudomonas* spp. and *Listeria* spp. were found to be present only in the sample *Phyllanthus emblica* (Gooseberry), *Ananas comosus* (pineapple), *Citrus sinensis* (malta) and *Spondias duscis* (ambarella) ( $\sim 10^2$  cfu/g). The presence of these pathogenic organisms revealed the possibility of spreading enteric diseases to the consumers. On the other hand, fungal load was observed in four samples, all were within the range of  $10^3$  cfu/g (Table 1). Presence of fungus indicates the presence of various mycotoxins in the fruit samples that may pose severe threat to the human health (33, 34).

**Anti-bacterial activity of the citrus fruit samples.** Chemically manufactured medicines may have numerous undesirable side effects on our body but natural antimicrobial agents from the fruits can be used safely in near future as anti-infective pharmaceutical products

TABLE 1. Microbial proliferation in citrus-based fruits samples (cfu/g)

Sample	TVB	Fungi	<i>Klebsiella</i> spp.	<i>Staphylococcus</i> spp.	<i>Pseudomonas</i> spp.	<i>Vibrio</i> spp.	<i>Listeria</i> spp.
<i>Phyllanthus emblica</i> (Gooseberry)	$1.9 \times 10^6$	$1.2 \times 10^3$	0	$5.6 \times 10^3$	$6.0 \times 10^2$	0	$9.0 \times 10^2$
<i>Prunus mume/domestica</i> (Plum)	$4.0 \times 10^2$	0	0	0	0	0	0
<i>Averrhoa carambola</i> (Carambola)	$2.9 \times 10^3$	0	0	0	0	0	0
<i>Tanarindus bacilus</i> (Tamarind)	$1.2 \times 10^4$	0	0	0	0	0	0
<i>Monifera indica</i> (Mango)	$6.3 \times 10^7$	$3.6 \times 10^3$	$1.9 \times 10^2$	0	0	$3.1 \times 10^2$	0
<i>Citrus limon</i> (Lemon)	$7.0 \times 10^6$	$1.9 \times 10^3$	$2.7 \times 10^2$	0	0	$1.8 \times 10^2$	0
<i>Phyllanthus acidus</i> (Otahe Gooseberry)	$7.8 \times 10^7$	$1.9 \times 10^3$	$2.2 \times 10^2$	0	0	$2.2 \times 10^2$	0
<i>Ananas comosus</i> (Pineapple)	$1.6 \times 10^7$	0	$5.0 \times 10^2$	$1.5 \times 10^2$	$8.0 \times 10^2$	0	$3.5 \times 10^2$
<i>Citrus sinensis</i> (Malta)	$1.8 \times 10^6$	0	0	$7.0 \times 10^2$	$9.0 \times 10^2$	0	$2.6 \times 10^2$
<i>Spondias duscis</i> (Ambarella)	$1.7 \times 10^7$	0	$2.0 \times 10^2$	$3.0 \times 10^2$	$2.0 \times 10^2$	0	$3.0 \times 10^2$

*Escherichia coli*, *Salmonella* spp. and *Shigella* spp. were totally absent in all the samples tested.

TABLE 2. Biochemical identification of different isolates

Assumed Organism	TSI			H <sub>2</sub> S Creation	Indole test	MR test	VP test	Citrate test	Motility	Oxidase test
	Slant	Butt	Gas							
<i>Klebsiella</i> spp.	Y	Y	+	-	-	-	+	+	-	-
<i>Vibrio</i> spp	Y	Y	-	-	+	+	-	+	+	+
<i>Pseudomonas</i> spp.	R	R	-	-	-	-	-	+	+	+
<i>Staphylococcus</i> spp.	Y	R	+	+	-	+	-	+	+	-
<i>Listeria</i> spp	Y	Y	-	-	-	+	+	-	+	-

without creating any bad effects in our body. Antimicrobial activity of various fruits such as guava, orange, dragon fruit, malta, apple, has been experimented around the world in different times (35-37).

Interestingly, in the present study, *Prunus mume* (plum) was found to show the anti-bacterial activity against the entire laboratory isolates of *Escherichia coli*, *Klebsiella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Salmonella* spp., *Vibrio* spp., and *Listeria* spp. (Table:3). Among other samples, *Tanarindus bacilus* (tamarind) exhibited activity against *Escherichia coli*, *Pseudomonas* spp., *Salmonella* spp., *Vibrio* spp., and *Listeria* spp. Whereas *Monifera indica* (mango) were found to be affective against *E. coli*, *Staphylococcus* spp. and *Listeria* spp. (Table: 3). A study conducted by Bansode and Chavan, 2012 (36) in India showed the presence of antimicrobial activity of *Citrus limon* (lemon) against some enteric pathogens such as *E. coli*, *Salmonella paratyphy* B and *Shigella sonnei*. Surprisingly, in this study no anti-bacterial activity of *Citrus limon* was found against *E. coli* and most of the other laboratory

isolates; however, it showed some degree of activity against *Pseudomonas* and *Listeria* spp. (Table 3). Reports on antimicrobial activity of *Phyllanthus acidus*, *Ananas comosus* and *Citrus sinensis* have also been shown by several authors (38, 39), but none of these three fruits showed any activity at all in this study. Variation in the antibacterial activity among the different citrus fruits may be due to inappropriate extraction of the active component of some of the samples. Nevertheless, the findings suggest that the citrus fruits may have the potential to play an antimicrobial role against many of the disease causing harmful pathogens.

## CONCLUSION

In fine, it can be concluded that the presence of a wide array of microorganisms with a huge load (near about 10<sup>7</sup> cfu/g) in the different fruit samples is a matter of great concern for the consumers. In addition, a number of pathogenic and indicator bacteria such as *Klebsiella* spp. in five samples, *Listeria* spp., *Staphylococcus* spp. and *Pseudomonas* spp. in four samples alongwith *Vibrio* spp. in three samples have been detected. Besides, fungal

TABLE 3. Antimicrobial activity of the citrus fruit samples

Sample	<i>E. coli</i>	<i>Klebsiella</i> spp.	<i>Staphylococcus</i> spp.	<i>Pseudomonas</i> spp.	<i>Salmonella</i> spp.	<i>Vibrio</i> spp.	<i>Listeria</i> spp.
<i>Phyllanthus emblica</i> (Gooseberry)	0	0	0	0	0	0	0
<i>Prunus mume</i> (Plum)	15mm	18mm	15mm	13mm	13mm	13mm	16mm
<i>Averrhoa carambola</i> (Carambola)	0	0	0	0	0	0	0
<i>Tanarindus bacilus</i> (Tamarind)	12mm	0	0	11mm	12mm	12mm	14mm
<i>Monifera indica</i> (Mango)	11mm	0	13mm	0	0	0	13mm
<i>Citrus limon</i> (Lemon)	0	0	0	12mm	0	0	13mm
<i>Phyllanthus acidus</i> (Otahe Gooseberry)	0	0	0	0	0	0	0
<i>Ananas comosus</i> (Pineapple)	0	0	0	0	0	0	0
<i>Citrus sinensis</i> (Malta)	0	0	0	0	0	0	0
<i>Spondias duscis</i> (Ambarella)	0	0	0	0	0	0	0
Positive control (Gentamicin, GEN- 10 µg)	25mm	26mm	27mm	26mm	25mm	26mm	27mm
Negative control (Normal saline)	0	0	0	0	0	0	0

growth was also observed in a number of samples. Further study is required to detect the antibiotic resistance pattern of the isolates detected from the commonly consumed citrus fruits which is of significance from the view point of public health. Another aspect of the present study was the detection of anti-bacterial activity of the samples against most of the laboratory isolates tested (within a zone of inhibition of minimum 11 mm to a maximum of 16 mm). This indicates that the citrus fruits can be a potential alternative to the conventional commercially available antibiotics found in the market. So different extraction methods need to be standardized to find out the best one before using citrus fruits as therapeutic agents. Every possible measure should be taken by the different regulatory bodies to ensure the safe consumption of fruits.

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