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Antimicrobial activity screening for three *Citrus* pulp extracts and phytochemical constituency profiling

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Abstract
Selective utilization of plant extracts as potential source of pharmaceutical agents has been increasing in recent years. Many of the plant extracts possess bio-active components which inhibit the growth of some of the Gram positive and Gram negative bacterial pathogens. Three different species of *Citrus (Citrus limon, Citrus aurantifolia* and *Citrus macroptera*) fruits pulp extracts were allowed to antimicrobial screening against pathogenic bacteria viz., *Staphylococcus aureus, Escherichia coli, Klebsiella sp., Pseudomonas sp. and Salmonella sp.* Ethanol extract of *Citrus macroptera* showed highest zone of inhibition (14±0.25mm) against *Klebsiella* sp. and methanol extract of *Citrus aurantifolia* showed highest zone of inhibition (8 ± 0.56 mm) against *Salmonella* Sp. Ethanol extract and methanol extracts of *Citrus* fruits are more effective against pathogenic bacteria. Among different extraction methods, water bath method showed better result than soaking method. So it could be assumed that *Citrus* fruit may release more bioactive compound in water bath method rather than soaking method. Qualitative phytochemical screening revealed the presence of various active phytoconstituents in the ethanolic extract of three *Citrus* species.

Keywords: *Citrus* sp., Secondary metabolites, antimicrobial activity, phytochemicals

Introduction
Fruits are essential part of diet and also known to ease the risk of several chronic diseases. The protective nature of fruits is due to the presence of phyto constituents such as poly phenolic compounds [1]. The *Citrus* is a fruit bearing plant of family Rutaceae and are cultivated worldwide [2, 3]. *Citrus* species are valued for its nutritional qualities, distinct flavor, and health benefits. The genus *Citrus*, includes 12 known species and great sources of bioactive compounds [4, 5]. Fruits of *Citrus* have valuable effects on human health due to the presence of vitamin C, carotenoids, flavonoids, limonoids, essential oils, acridone alkaloids, minerals, vitamin B complex [6, 7] and popular in food, drug, and cosmetic industries due to their medicinal properties and fragrance.
Citrus limon is commonly known as lemon is an important medicinal plant used mainly for secondary metabolites which has anticancer and antibacterial activities [8, 9]. Besides, *Citrus aurantifolia* commonly known as lime, is native to Southern Asia and cultivated in the West Indies, semi-tropic areas of the U.S. and Central America [10, 11]. Existing literatures on the antimicrobial activities of lime oil states its potent antibacterial [12, 13] and antifungal effects [14, 15] where lime essential oil has several medicinal properties (presence of secondary plant metabolites including flavonoids, coumarins, and terpenoids) and potential health benefits which makes it a good candidate as a natural antimicrobial preservative in food products. The other important *Citrus* species is *Citrus macroptera* var. anamnesis, locally known as "Satkara" in Bengali and "Wild orange" in English [16, 17] is grown in the yard of most homesteads and hill tracts of the Sylhet division of Bangladesh. The fruit is typically used during cooking and for pickle preparation and is popular for its medicinal purposes [18, 19]. The fruit has significant cytotoxic, antimicrobial, antihypertensive, antipyretic, and appetite stimulant potentials [20, 21, 22]. Additionally, significant hypoglycemic and neuro pharmacological effects were confirmed in a rat model [23, 24]. The antioxidant constituents in the *C. macroptera* fruit include phenolics, flavonoids, tannins, ascorbic acid, and proteins, and significant radicals scavenging activity was confirmed by an *in vitro* study [25, 26].
However, *Citrus* species are still neglected for investigation of pharmacognosical potential in Bangladesh. In the present investigation, our aim was to evaluate two different extraction methods using methanol, ethanol, acetone and distilled water followed by antimicrobial activity test against five bacterial stains for three *Citrus* fruit Species of Bangladesh.

Materials and methods

Collection and preparation of fruit pulps

Fruits of three different species of Citrus plants, *Citrus limon*, *Citrus aurantifolia* and *Citrus macroptera* were collected from Jaintapur region located at Sylhet, Bangladesh for the present study. The samples were then preserved at standard condition in the Biotechnology and Genetic Engineering lab, Faculty of Biotechnology & Genetic Engineering, Sylhet Agricultural University, Sylhet for the experiments. To process samples, fruits were thoroughly washed to remove dirt and other materials. The fruit pulps were cut into small pieces and dried at sunlight to reduce moisture contents to the minimal. Clean dried samples were grounded using an electronic blender and subjected for extraction.

Preparation of crude extracts from fruit pulp

Crude extracts were prepared from *Citrus pulp* by following two methods (Soaking and water bath) using distilled water, 80% ethanol, methanol and acetone. Briefly, in case of soaking method, 10 grams of each powdered *Citrus pulp* samples were soaked in a conical flask containing 80 ml of the distilled water, 80% ethanol, methanol and acetone. The samples were gently mixed by shaking. And left for 72 hours in room temperature. The liquid phase was then filtered with Whatman no. 1 filter paper. In case of water bath method, five grams powdered *Citrus pulp* samples were soaked in a conical flask containing 40 ml of the distilled water, 80% ethanol, methanol and acetone. Then gently mixed with shaking. Samples were heated for 4 hours at 50^0C temperature. The liquid phase was then filtered with Whatman no.1 filter paper. The filtered liquid phase was allowed to dry to make distilled water, ethanol, methanol and acetone evaporate and dried extract were remained at the bottom. Dried extracts were collected.

Antimicrobial activity test

This test was done using the disc diffusion method (also known as Kirby-Bauer method) [27]. Briefly, pure cultures of five different bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella*, *Pseudomonas*, *Salmonella*) were used. To prepare test plates, each plate was poured with 15 ml of Muller-Hinton agar medium. Blank discs were prepared by using filter paper. Dried and sterilized blank discs were treated separately with test solution. After preparation, discs were placed into center of an agar plate by using a sterile forceps and pressed down. The plates were then inverted and incubated at 37ºC within 15 minutes after the discs were applied. After 24 & 48 hours of incubation, each plate was examined.

Analysis of zone ratio

There were uniformly circular zone of inhibition on the surface. The diameter of the complete zone of inhibition (Judged by unaided eye) was measured including the diameter of the discs. Zones were measured to nearest whole millimeter, using a ruler.

Preliminary phytochemical evaluation

Preliminary Phytochemical evaluation was performed on the ethanol extract of pulp powder of three different *Citrus* species by following standard methods as described by Treaseand Evans, Sofowra, Harborne, Howlader *et al*. and Ahmed *et al*. [28, 29, 30, 31, 32].

Results

Antimicrobial Activity of *Citrus pulp* extracts by soaking method

In soaking method, we used different solvents such as ethanol, methanol and acetone. Antimicrobial Activity of *Citrus* fruit pulp extracts by soaking method was demonstrated at Table-1.

Table 1: Screening of antibacterial activity of *Citrus pulp* extracts by soaking method

Organic Solvent (80%)	Bacterial Isolates	Concentration of bacterial culture	Zone of inhibition (mm)			
			Citrus limon	*Citrus macroptera*	*Citrus aurantifolia*	Gentamicin (Control)
Ethanol	*S. aureus*	50 µL	5 ± 0.75	5±0.30	5± 0.88	18 ± 0.89
	E. coli	50 µL	5 ± 0.28	5±0.40	5 ± 0.76	19 ± 0.23
	Klebsiella sp.	50 µL	5 ± 0.30	14±0.25	5 ± 0.45	15 ± 0.19
	Pseudomonas sp.	50 µL	5 ± 0.80	5±0.60	5± 0.86	17 ± 0.94
	Salmonella sp.	50 µL	5 ± 0.35	5±0.75	5± 0.28	18 ± 0.75
Methanol	*S. aureus*	50 µL	6 ± 0.50	5±0.55	5± 0.35	18 ± 0.25
	E. coli	50 µL	5 ± 0.80	5±0.60	5 ± 0.90	19 ± 0.50
	Klebsiella sp.	50 µL	5 ± 0.75	5±0.90	5 ± 0.36	15 ± 0.41
	Pseudomonas sp.	50 µL	5 ± 0.90	6±0.20	15 ± 0.65	17 ± 0.34
	Salmonellas sp.	50 µL	5 ± 0.85	5±0.60	5 ± 0.63	18 ± 0.40
Acetone	*S. aureus*	50 µL	6 ± 0.26	5±0.15	5 ± 0.55	18 ± 0.28
	E. coli	50 µL	5 ± 0.38	5±0.50	5 ± 0.50	19 ± 0.56
	Klebsiella sp.	50 µL	5 ± 0.90	5±0.20	5± 0.32	15 ± 0.74
	Pseudomonas sp.	50 µL	5 ± 0.57	5±0.40	5 ± 0.28	17 ± 0.52
	Salmonella sp.	50 µL	5 ± 0.63	5±0.50	5 ± 0.30	18 ± 0.46

Data are the means of three replicates (n=3) ± standard deviations.

Antimicrobial activity of *Citrus pulp* extracts by Water Bath method

Water bath method, we used different kinds of solvents. There is no any significance performance in water bath method. Antimicrobial activity of *Citrus* fruit extracts by Water Bath method illustrated in Table-2.

Table-2: Screening of antibacterial activity of *Citrus pulp* extracts by water bath method

Organic solvent (80%)	Bacterial Isolates	Concentration of bacterial culture	Zone of inhibition (mm)			
			Citrus limon	*Citrus macroptera*	*Citrus aurantifolia*	Gentamicin (Control)
Ethanol	*S. aureus*	50 µL	5 ± 0.28	5± 0.40	5 ± 0.77	18 ± 0.89
	E. coli	50 µL	6 ± 0.57	7± 0.26	5 ± 0.45	19 ± 0.23
	Klebsiella sp.	50 µL	5 ± 0.25	5± 0.45	5 ± 0.66	15 ± 0.19
	Pseudomonas sp.	50 µL	5 ± 0.56	7± 0.58	5 ± 0.89	17 ± 0.94
	Salmonella sp.	50 µL	6 ± 0.45	7± 0.60	5 ± 0.52	18 ± 0.75
Methanol	*S. aureus*	50 µL	5 ± 0.89	7± 0.50	5 ± 0.35	18 ± 0.25
	E. coli	50 µL	6 ± 0.76	7± 0.48	5 ± 0.83	19 ± 0.50
	Klebsiella sp.	50 µL	5 ± 0.23	5± 0.70	5 ± 0.64	15 ± 0.41
	Pseudomonas sp.	50 µL	5 ± 0.43	5± 0.34	7 ± 0.86	17 ± 0.34
	Salmonella sp.	50 µL	8 ± 0.27	6± 0.49	8 ± 0.56	18 ± 0.40
Acetone	*S. aureus*	50 µL	5 ± 0.48	7± 0.56	5 ± 0.47	18 ± 0.28
	E. coli	50 µL	6 ± 0.37	6± 0.30	5 ± 0.67	19 ± 0.56
	Klebsiella sp.	50 µL	5 ± 0.65	5± 0.35	5 ± 0.84	15 ± 0.74
	Pseudomonas sp.	50 µL	5 ± 0.39	6± 0.25	7 ± 0.11	17 ± 0.52
	Salmonella sp.	50 µL	7 ± 0.43	8± 0.50	7 ± 0.66	18 ± 0.46

Data are the means of three replicates (n=3) ± standard deviations.

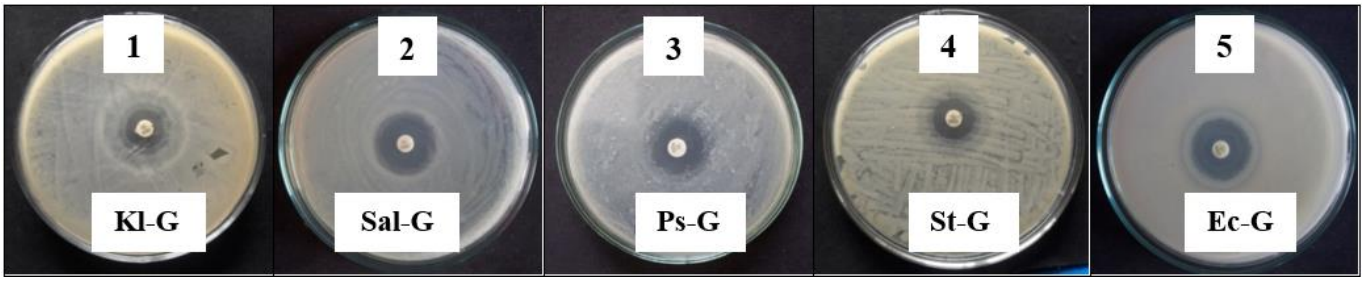

Fig 1: Growth inhibition zone of Gentamicin against 1. *Klebsiella* sp., 2. *Salmonella* sp., 3. *Pseudomonas* sp. 4. *Staphylococcus aureus* and 5. *E. coli*

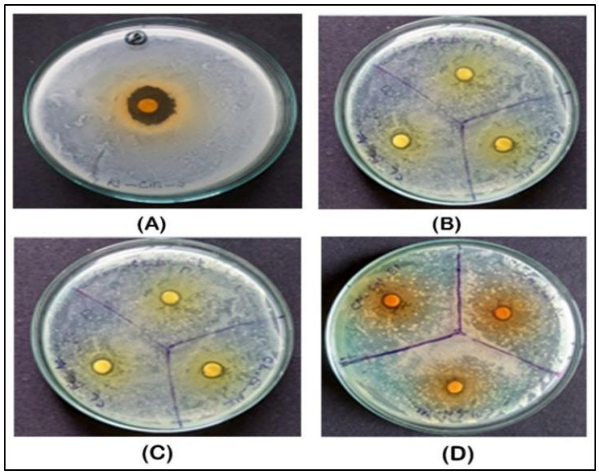

Fig 2: Antibacterial activity screening of *Citrus pulp* extracts; A) Growth inhibition zone of *C. macroptera* ethanol extract (soaking method) against *Klebsiella* sp. B) Growth inhibition zone of *C. limon* ethanol, methanol and acetone extract (soaking method) against *E. coli* C) Growth inhibition zone of inhibition *C. macroptera* ethanol, methanol and acetone extract (water bath method) against *Salmonella* sp. D) Growth inhibition zone of inhibition *C. macroptera* ethanol, methanol and acetone extract (water bath method)against *E. coli.*

Preliminary phytochemical evaluation

Different phytochemical tests confirmed the presence of various active phytoconstituents in three different Citrus species that are presented in Table 3.

Table 3: Preliminary Phytochemicals analysis of three Citrus species

Test of Phytochemicals	*Citrus limon*	*Citrus macroptera*	*Citrus aurantifolia*
Alkaloids	+	+	-
Tannins	+	+	+
Phenolic compounds	+	+	+
Anthraquinones	-	-	+
Flavonoids	-	+	+
Terpenoids	+	-	+
Steroids	+	-	+
Saponins	-	-	-
Cardiac glycosides	+	+	+
(+) means presence and (-) means absence			

Discussion

Infectious diseases are the major cause of morbidity and mortality worldwide. The number of multidrug resistant microbial strains are appeared which reduces susceptibility to antibiotics are continuously increasing. Such increase has been attributed to indiscriminate use of broad spectrum antibiotics. This situation provides a scope to the search for new antimicrobial substances from various sources like medicinal plants. However, the antibacterial activity of three Citrus fruits pulp extracts (*Citrus limon*, *Citrus aurantifolia* and *Citrus macroptera*) were analyzed against four gram negative (*Escherichia coli*, *Klebsiella* sp., *Pseudomonas* sp., *Salmonella* sp.) and one gram positive (*Staphylococcus aureus*) bacteria [33]. In the current study we used different solvent to extract citrus fru5t samples such as ethanol, methanol, acetone. Ethanol extract of *Citrus macroptera* showed highest zone of inhibition (14±0.25mm) against *Klebsiella* sp. And methanol extract of *Citrus aurantifolia* showed highest zone of inhibition (8 ± 0.56 mm) against *Salmonella* Sp. Ethanol extract and methanol extracts of *Citrus* fruit extracts are more effective against pathogenic bacteria. In our current study, two different extraction methods were used. Among them, *Citrus* fruit extracts showed better results in water bath extraction method than soaking method. So it could be assumed that *Citrus* may release more bioactive compound in water bath method rather than soaking method. It was observed that pulp oil of *C. sinensis* and *C. aurantium* exhibited more or less similar inhibition of Gram positive and Gram negative bacteria [34, 35]. In our current study *Citrus* fruit more or less effective against gram positive and gran negative bacteria. The study of Siddique *et al*. showed the efficacy of essential oil from pulp of *C. aurantium* to inhibit Gram positive bacteria but not Gram negative bacteria [36]. Tumane *et al*. observed marked inhibitory effect of ethanolic and methanolic extract of *C. aurantium* against a panel of bacteria [37]. The antibacterial potential of the crude extracts against clinically significant bacterial strains has also been reported [38, 39]. Exhibits little antimicrobial activity due to presence of these secondary metabolites [40, 41]. Qualitative screening test for phytochemicals revealed that tannin, phenolic and cardiac glycosides were present in all *Citrus* species whereas saponins were absent in all *Citru*s species and other phytochemicals showed varied presence among Citrus species. Result noticed the presence of anthraquinones only in *Citrus aurantifolia*.

Conclusion

The plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being. Secondary metabolites are organic compound produced by plant but not involved in the growth and development process of the plant but act as inhibitor of microorganisms. Antimicrobial activity of pulp extract is directly concerned with bioactive compounds that they contain which also indicates the presence of metabolic toxins or broad spectrum antibiotic compounds.

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