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Potent Inhibition of swarming growth of Uropathogenic *Proteus* Bacteria by Ethanolic extracts of *Emblica officinalis* fruit and *Tamarindus indica* bark

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Abstract

Medicinal plants have been the principal source for most of the drugs. Phytochemicals having antimicrobial properties can be of great significance in therapeutic treatments. Several pathogenic features of uropathogenic Proteus species isolated from municipal water have earlier been studied. Strong resistance to cephalosporin was found in those strains. This study aimed to evaluate the role of medicinal plants to interfere with the growth and virulence of those strains as an alternative therapy of UTI. Six strains with high pathogenicity were undertaken to a test of bactericidal activity by normal human serum (NHS). Then NHS resistant strains were targeted to combat them by phytochemicals. Five medicinal plants, Emblica officinalis, Asparagus racemosus, Azadirachta indica, Abroma augusta, and Tamarindus indica, were selected for the purpose. Four strains named 11(Pv), 91_1(Pm), 91_2(Pm), 66_1(Pp) were resistant to NHS being 91_2(Pm) the strongest. Ethanol extracts of A. augusta had no anti-Proteus activity and that of A. indica, A. racemosus showed noticeable inhibition of Proteus cells. However, very low concentration of extracts of E. officinalis (400µg/ml) and T. indica (25µg/ml) completely inhibited the swarming although they had essentially no effect on cell proliferation. Ethanol extracts of E. officinalis and T. indica are potent inhibitor of Proteus swarming. Edible fruit (E. officinalis) known as Indian gooseberry/Amla can be used as food supplement to cure UTI and to control bacterial pathogens having swarming abilities.

Keywords: Alcoholic extract, *Emblica officinalis*, swarming inhibitor, *Tamarindus indica*.

Introduction

Herbal care or conventional systems of medicine have been using from ancient times. Medicinal plants especially herbs have been the original source for most of the drugs. Now-a-days about 70% of the world population is depending on medicinal herbs. Medicinal plants contain so many chemical compounds which are the major source of therapeutic agents to cure human diseases[1]. For a long period of time, medicinal plants have been the valuable source of natural products for maintaining human health, especially in the last decade, with more intensive studies for natural therapies. The use of phytochemicals for pharmaceutical purposes has been gradually increased.

According to World Health Organization the medicinal plants could be the best source to obtain a variety of drugs. Approximately 80% people in developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety and efficiency[2]. The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency[3-7]. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. The antimicrobial properties of medicinal plants have been investigated by many investigators worldwide, especially in Indian region. Thirty one medicinal plant species have been reported by traditional healers as being used for UTIs, including leucorrhea, frequent or infrequent urination, cloudy urination, and burning sensations during urination in Bangladesh[8].

The *Proteus* pathogens are thought to be the principal cause of UTI, CAUTI and wound infections. We isolated pathogenic *Proteus* bacteria from municipal tap water[9] that were multidrug resistant especially to cephalosporin,[10] and several pathogenic features of those isolates have already been reported[11-12]. The increasing evidence of antibiotic resistance in bacterial pathogens necessitates medicinal plants as an alternative therapy in restricting the antibiotic resistant infectious organisms. We selected here five medicinal plants having potential antimicrobial properties that are traditionally used as folk medicine for urological disorder. Leaves of *Abroma augusta* Linn (Family: Malvaceae) has been widely investigated and its antibacterial potentials have been reported by researchers[13-14]. *Emblica officinalis* Gaertn (Family: Phyllanthaceae) is commonly known as Indian gooseberry or amla. The alcoholic extract of *E. officinalis* exhibited strong and broad spectrum antibacterial activity against various pathogenic bacteria and numerous biological activities has also been reported[15-17]. This tree grown or cultivated in subtropical and tropical parts of China, India, Indonesia and the Malay Peninsula has been used for anti-inflammatory and antipyretic treatments of rural populations in these areas[18]. The root extract of *Asparagus*

racemosus (Family: Asparagaceae) which is locally known as shotomuli showed antibacterial activity against resistant uropathogens isolated from patients having UTI[19]. The alcoholic extract of *Azadirachta indica* leaf (Family: Meliaceae) which is locally named neem showed potential antimicrobial activities including *Proteus mirabilis*[20]. Nwodo et al. 2011 found the significant antimicrobial activities in aqueous and alcoholic extract of *Tamarindus indica* bark (Family: Leguminosae)[21].

Therefore, this study aimed to investigate the role of ethanolic fraction of test medicinal plants to interfere with the growth and virulence of cephalosporin resistant uropathogenic *Proteus* bacteria isolated from municipal supplied water in our previous study.

Material and Methods

Bacterial strains: Six *Proteus* strains belonging four species i.e. *P. vulgaris* (hereafter termed as *Pv*), *P. mirabilis* (*Pm*), *P. hauseri* (*Ph*), and *P. penneri* (*Pp*) designated as 11(*Pv*), 66_1(*Pp*), 66_2(*Ph*), 66_3(*Pp*), 91_1(*Pm*) and 91_2(*Pm*) isolated from municipal tap water (Rajshahi City, Bangladesh) in our previous study[9] have been used. Those strains were multidrug resistant to broad spectrum antibiotics and possessed several pathogenic features including swarming motility, urease production, extracellular proteases, biofilm formation as reported earlier[10-12]. Strains stored at −40ºC in Luria-Bertani (LB) broth supplemented with 12% (v/v) glycerol were freshly grown at 37ºC to carry out this study.

Growth media and culture conditions: Nutrient agar media (1% yeast extract, 0.5% peptone, 0.5% NaCl, 1.5% agar) purchased from Difco was used for antibacterial activity assay of the extracts. The strains were incubated at 37°C for overnight as described elsewhere[9-10]. Cells were cultured in nutrient broth media with mild shaking on a water bath (Advantec Lab-Thermo Shaker, TS-20, Toyo Kaisha, Ltd) at 37°C to test the growth rate of bacterial cell.

Swarming motility test: In 10ml of LB broth medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) *Proteus* strains were grown overnight at 37ºC with shaking (200 rpm). Then 5µl of fresh cell culture was spotted at the center of LB agar plates (LB medium containing 1.5% agar) previously dried to remove water drops from the surface of the agar medium as described in other reports[22] and incubated at 37ºC for 24 hrs unless it is mentioned otherwise. Then the diameters of swarming zones were measured in millimeter at three different directions.

Plant material: Plant parts were collected from the medicinal plant garden, Department of Pharmacy, University of Rajshahi and around Rajshahi City area, Bangladesh on Nov 2013, and duly identified by a plant taxonomist Mr. Arshed Alom, Department of Botany, University of Rajshahi, Bangladesh where a specimen voucher (75/05.07.2008) was recorded in the department herbarium for future reference. Plant parts i.e. fruit of *Emblica officinalis* Gaertn (Family: Phyllanthaceae, local name: Amla/Amloki), root of *Asparagus racemosus* (Family: Asparagaceae, local name: Shotomuli), leaf of *Azadirachta indica* (Family: Meliaceae, local name: Neem), bark of *Tamarindus indica* (Family: Leguminosae, local name: Tentul), leaf of *Abroma augusta* Linn (Family: Malvaceae, local name: Ulotcombol) were air-dried under shade. Once dried, the plant material was ground, extracted by maceration for 48-72 hours with ethanol, filtered (Paper Whatman No. 3) and the solvent was vacuum evaporated in a Soxhlet apparatus (Rotary Evaporator, RE 300, Bibby Sterilin Ltd, UK). Then solutions were evaporated to dryness and further dilutions were made in the same solvent to obtain the required extract concentrations for the different assays.

Table-1
Anti-*Proteus* activity of ethanolic extracts of five medicinal plants

Proteus **strain (spp)**	**Disk potency (500 µg/disk)**					**Cotrimoxazole** (5 µg/disk)
	A. indica	*A. augusta*	*A. racemosus*	*T. indica*	*E. officinalis*	
11(*Pv*)	17 ± 1.14	–	14 ± 1.12	7 ± 0.07	12 ± 0.22	34 ± 0.84
66_2(*Ph*)	18 ± 0.38	–	15 ± 0.13	7 ± 0.03	11 ± 0.98	28 ± 1.21
91_1(*Pm*)	12 ± 0.19	–	13 ± 0.09	6 ± 0.08	8 ± 0.88	30 ± 0.22
91_2(*Pm*)	15 ± 0.51	–	14 ± 0.22	7 ± 0.54	9 ± 0.78	30 ± 1.11
	Disk potency (1 mg/disk)					
	A. indica	*A. augusta*	*A. racemosus*	*T. indica*	*E. officinalis*	
11(*Pv*)	18 ± 0.28	–	16 ± 0.82	7 ± 0.76	16 ± 0.87	34 ± 1.26
66_2(*Ph*)	15 ± 0.98	–	14 ± 0.88	8 ± 0.22	13 ± 0.34	28 ± 1.09
91_1(*Pm*)	15 ± 0.78	–	15 ± 0.25	8 ± 0.43	11 ± 1.09	30 ± 0.24
91_2(*Pm*)	17 ± 1.21	–	14 ± 0.99	8 ± 0.34	11 ± 0.76	30 ± 0.98

Sign (−) indicates no zone of inhibition detected. *A. indica* leaf and *A. racemosus* root showed notable zone of inhibition but *T. indica* bark and *E. officinalis* fruit showed either no or very low zone of inhibition compared to that of reference

Test for antibacterial activity: Each of plant extracts was tested for its anti-*Proteus* activity following disk diffusion method on nutrient agar media[23-25]. The crude extracts were separately dissolved in 1 ml of ethanol and the filter paper discs (6 mm diameter) were impregnated with known amounts of test substances and prepared in various potencies, 25µg to 1 mg/disc. Discs were placed on pre-seeded bacterial culture plate of *Proteus* strain by sterilized forceps. Plates were then kept overnight at 4°C to allow maximum diffusion of components of disc. The plates were then incubated at 37°C for 18 hrs. Then the diameter (in millimeter) of zone of inhibition for each extract against tested microorganisms was noted. Reference standard disc of cotrimoxazole (5µg, Hi-media, India) was used as positive control and blank disc impregnated with solvent followed by drying off was used as negative control. All tests were performed in triplicate and the antibacterial activities were calculated by measuring the diameter of zone of inhibition.

Inhibition of swarming motility: Effects of plant extracts on swarming motility of isolates were assessed as described in other report[26]. Briefly, an overnight bacterial culture (5µl) was inoculated centrally onto the surface of dry LB agar plates made with or without extracts at various concentrations which were then incubated at 37ºC for 24 hours. The perimetric distance of swarming motility was assayed by measuring the fronts of swarming areas in three different directions.

Normal human serum (NHS) preparation : The whole blood from five healthy university students was collected in a test tube at university health care center. After collection, the blood was allowed to clot leaving undisturbed at room temperature. The blood clot was removed by centrifugation at 6000 rpm for 20 minutes in a refrigerated centrifuge. The resulting supernatant was designated as serum that apportioned into 0.5 ml aliquots, stored at –20°C. Heat inactivation, i.e. the destruction of complement activity was achieved by incubating the serum for 1 h at 56°C before use in bactericidal activity[27].

Bactericidal activity of NHS: 50µl of standardized (~10^9 CFU/ml) fresh bacterial cell suspension adjusted with physiological saline (0.9% NaCl) was immediately mixed with equal volume of NHS and incubated at 37ºC for 3 hours while samples were pooled at 0, 0.5, 1, 2 and 3 h of incubation to test the bactericidal activity of NHS against multidrug resistant *Proteus* strains. Then the samples plated on nutrient agar medium were subjected to the colony counting process by serial dilution method. The colony count of pooled samples was scored as percent of bactericidal activity regarding the value at 0 h as 100%. Those strains were regarded as resistant to bactericidal activity of NHS whose survival in the serum was 50% after 3 h of incubation[22, 28-29].

Data analysis: For data processing, the software Microsoft Excel 2007 was used. Results of triplicate experiments were averaged, and means ± standard deviations were calculated.

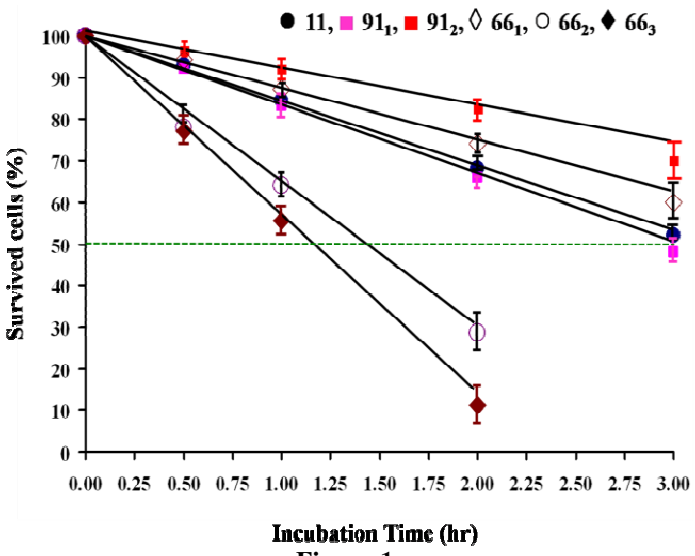

Figure-1
Bactericidal activity of NHS to *Proteus* isolates
The slope of each linear line indicating the rate of bactericidal activity of NHS to the respective strain are 0.164 (91_2), 0.227 (66_1), 0.302 (11), 0.322 (91_1), 0.720 (66_2) and 0.866 (66_3), Strains whose 50% cells can survive up to 3 hrs of incubation were regarded as NHS resistant

Results and Discussion

Bactericidal activity of NHS against Proteus strains: Six *Proteus* isolates were selected from eleven others based on their species and the extent of pathogenicity depending on few pathogenic factors, i.e. swarming motility, antibiotic resistance, extracellular proteases, biofilm forming abilities. Two strains, 91_2(*Pm*) and 66_2(*Ph*), had strong swarming abilities as reported earlier[11]. Strain 91_2(*Pm*) also showed strong proteolytic activity measured by well diffusion assay on casein agar plate[11]. Strains 11(*Pv*), 91_1(*Pm*), 66_1(*Pp*) were biofilm former on PVC strip as well as catheter strip[12]. These strains were subjected to a bactericidal activity assay with NHS as described in materials and methods section. The ability of NHS to kill the *Proteus* strains was different in each isolate. The decline of the number of survived cells with time which indicated the bactericidal activity of NHS was found to be rapid in the strains 66_2(*Ph*) and 66_3(*Pp*) (figure 1) whereas 50% bacterial cells of four other strains, 11(*Pv*), 91_1(*Pm*), 91_2(*Pm*), 66_1(*Pp*) were able to survive in the presence of NHS up to 3 h of incubation at 37°C. The result indicated that these four strains were resistant to NHS. Based on the rate of bactericidal activity of NHS, calculated from the slope of linear plot, the most resistant strain was 91_2(*Pm*) followed by 66_1(*Pp*), 11(*Pv*) and 91_1(*Pm*). This result supports our previous conclusion[11] where 91_2(*Pm*) showed the best proteolytic activity on casein agar plate and 66_1(*Pp*) was the second highest scorer. Therefore, we assumed that the secretion of extracellular proteases especially metalloprotease ZapA was high enough to combat against NHS for its survival.

Anti-Proteus activity of selected medicinal plants: Next we attempted to kill these NHS resistant *Proteus* strains by phytochemicals and to do so five medicinal plants, *T. indica* bark, *E. officinalis* fruit, *A. indica* leaf, *A. augusta* leaf, *A. racemosus* root, were selected based on their reported antibacterial activities against *Proteus* species[11,30-31]. The aqueous extract of *E. officinalis* fruit pulp is found to be effective against various pathogenic Gram-positive and Gram-negative bacterial strains including *Proteus* species[32]. This may be due to the presence of certain tanin, alkaloids and phenolic compounds present in the fruit extract of *E. officinalis*[33]. Here, we used the ethanolic extract of all plant parts to combat against NHS resistant uropathogenic *Proteus* strains (figure 2). Only the extract of *A. indica* and *A. racemosus* showed remarkable zone of inhibition by disk diffusion method (table 1) but the extract of *A. augusta* showed no zone of inhibition and that of *T. indica* and *E. officinalis* had very low or no activities (figure 2, table 1). Then the extracts were screened for their effects on swarming motility of the strains since swarming is one of the crucial pathogenic factors of *Proteus* bacteria.

Effects on swarming motility: *Proteus mirabilis* is thought to be the most pathogenic and opportunistic bacteria among other *Proteus* species. In our previous investigation, isolate 91_2(*Pm*) was found to be a strong swarmer, biofilm former and protease producer cell[10-12]. However, in this study the plant extracts exhibiting antibacterial activity were tested on the swarming growth of NHS resistant strains of 91_1(*Pm*) and 91_2(*Pm*). These two strains were undertaken to a study targeting their management and control by phytochemical(s) where cells were allowed to swarm onto the LB agar medium containing plant extracts at 500 µg/ml concentration (figure 3). It supposed that the *A. indica* extract had no effect on swarming growth but *A. recemosus* extract was found to show remarkable anti-swarming activity compared to that of control (figure 3) and especially the effect was pronounced for strain 91_2(*Pm*). Moreover, interestingly the extract of *E. officinalis* (figure 3) and *T. indica* (data not shown) completely inhibited the swarming growth at 400µg/ml concentration although their antibacterial activities were very low by disc diffusion method (table 1).

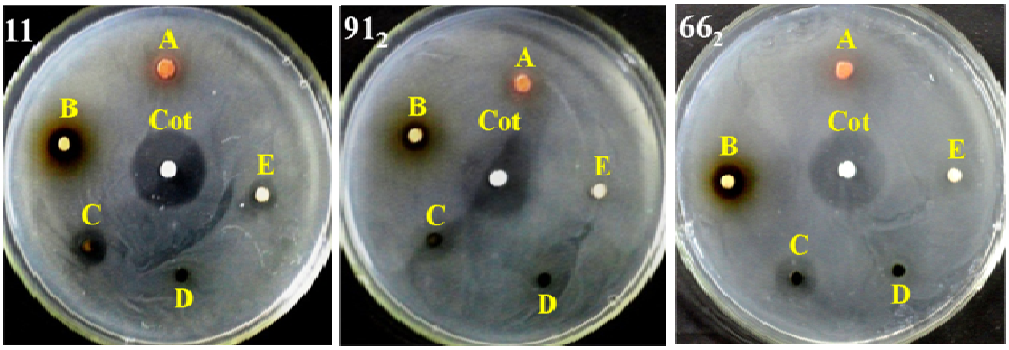

Figure-2
Anti-*Proteus* activity of plant extracts
Extracts at 1mg/disk concentration were used. A: *T. indica* bark, B: *E. officinalis* fruit, C: *A. indica* leaf, D: *A. augusta* leaf, E: *A. racemosus* root, Extracts of *A. indica* leaf and *A. racemosus* root showed remarkable activities

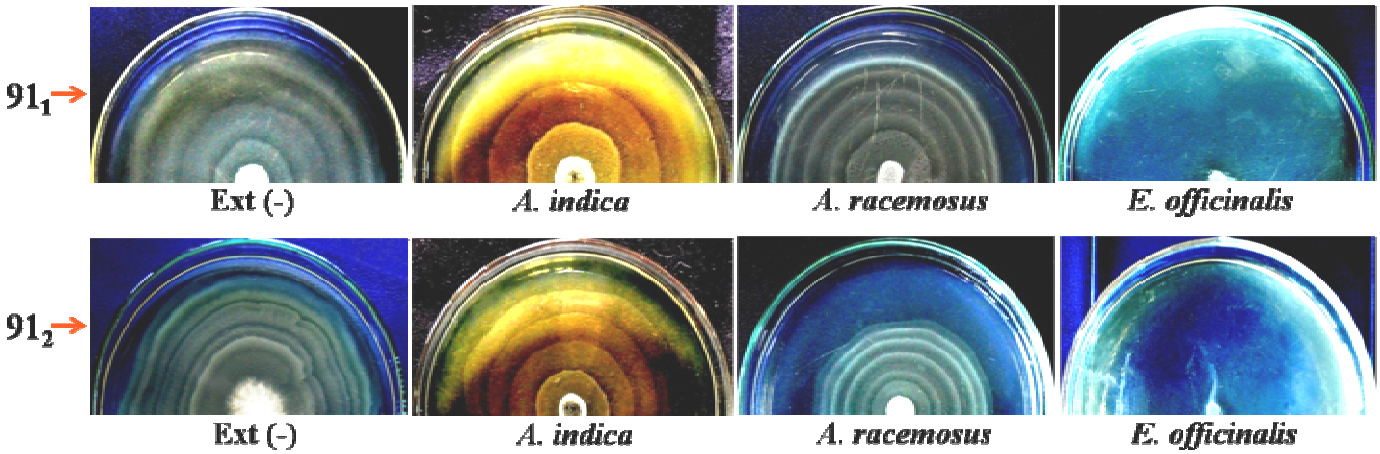

Figure-3
Inhibition of swarming motility of *P. mirabilis*
Two test strains were allowed to swarm over the surface of LB agar plate at 37°C for 24 hrs in the presence (*A. indica*, *A. racemosus*, *E. officinalis*) and absence (Ext−) of extracts, No swarming was found for the extract of *E. officinalis*

Swarming profile with time: Here the incubation time dependent swarming abilities of the test strains 91_1(*Pm*), 91_2(*Pm*) and 66_2(*Ph*) were investigated in the presence of *A. indica*, *A. racemosus* and *E. officinalis* extracts at 500 µg/ml concentration (figure-4). *A. indica* extract accelerated the swarming abilities of the test strains 91_1(*Pm*) and 66_2(*Ph*) within 3 h of incubation and ~2 fold acceleration was found at 9 h of incubation (figure 4A, C) whereas *A. racemosus* extract accelerated the swarming of 91_1(*Pm*) and 66_2(*Ph*) (figure 4A,C) but inhibited the swarming of 91_2(*Pm*) (figure 4B). Interestingly, at the same concentration *E. officinalis* extract completely inhibited the swarming of all test strains (figure 3, 4). Here, strain 91_2(*Pm*) exhibited faster swarming than two others, 91_1(*Pm*) and 66_2(*Ph*), resembling and strengthening our previous investigation[11] where strain 91_2(*Pm*) showed highest swarming among eleven others.

Table-2
Effects of extracts on cell proliferation

Extracts	Incubation time (h)									
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Control	0.030	0.041	0.085	0.198	0.458	0.551	1.045	1.442	1.759	2.122
E. officinalis	0.032	0.043	0.221	0.572	0.810	1.063	1.101	1.461	1.611	2.083
T. indica	0.034	0.046	0.240	0.575	0.821	1.145	1.310	1.541	1.714	2.212

20ml of LB broth medium was inoculated with 50µl of an overnight culture of 91_2(*Pm*) strain in the presence (*E. officinalis*, *T. indica*) and absence (Control) of extracts at 500µg/ml concentration. OD_{600nm} of pooled culture at indicated time was measured. No effects were observed on cell proliferation by the extracts.

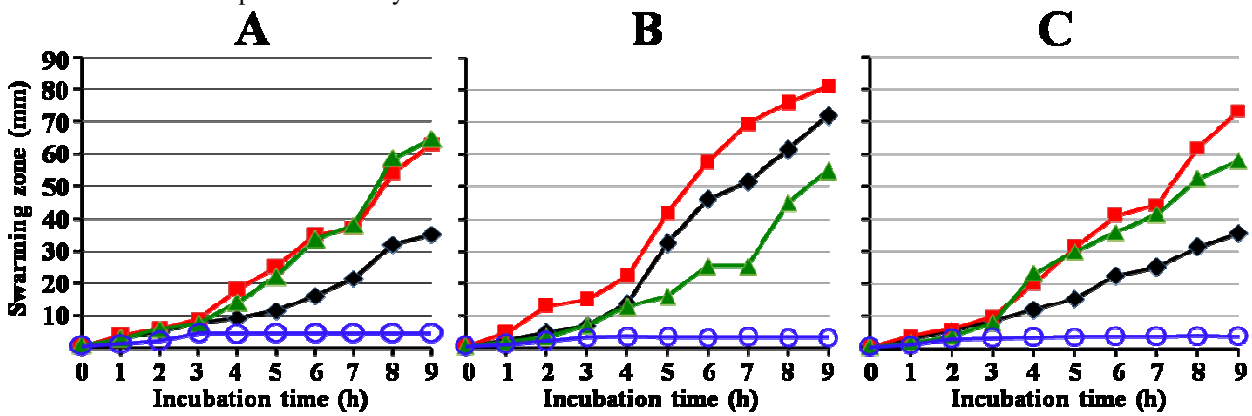

Figure-4
Effect of plant extracts on swarming over time
Three strains; A: 91_1, B: 91_2, C: 66_2, were allowed to swarm on LB agar medium in the absence (♦) and presence (500µg/ml) of plant extracts; *A. indica* (■), *A. racemosus* (▲), *E. officinalis* (○). Extract of *E. officinalis* inhibited the swarming of all test strains

Now to clarify the confusion whether the effect of extract is anti-bacterial or anti-swarming, we carried out additional experiment to test effects of extracts on cell proliferation in LB broth medium (table 2). The degree of cell proliferation with time of strain 91_2(*Pm*) was exponential in the absence or presence of extracts at 500µg/ml concentration up to 9 h of incubation resembling with the growth of wild-type *Pm* in the presence of resveratrol, a swarming inhibitor of *Pm*[34]. This result indicated that the test extracts had potential anti-swarming effect rather than the antibacterial activity. Then we aimed to determine the minimum concentration of the extract to inhibit swarming by disc diffusion method (figure 5). Clear zone of swarming was seen at ≥400µg of *E. officinalis* extract (figure 5A) and at ≥25µg of *T. indica* extract (figure 5B) by disc diffusion method which is essentially comparable to resveratrol (60µg/ml). Finally, the study led us to summarize that ethanolic fractions of *E. officinalis* fruit and *T. indica* bark are potent inhibitor of *Proteus* swarming motility.

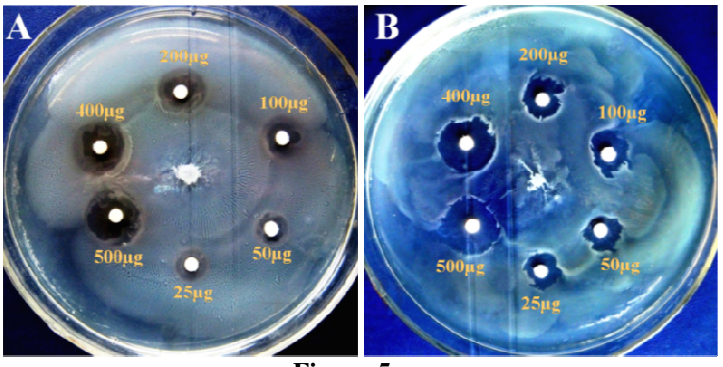

Figure-5
Concentration dependent inhibition of swarming by extracts
Strain 91_2(*Pm*) was allowed to swarm on LB agar plate at 37°C and paper discs containing various concentrations of extract of *E. officinalis* (A) and *T. indica* bark (B) were placed to test the minimum amount of extract enabling the inhibition of swarming that were ≥400µg for *E. officinalis* (A) and 25µg for *T. indica* (B)

Discussion: Among the common medicinal plants *E. officinalis* fruit commonly known as gooseberry and amla/amloki in Bangladesh plays a crucial role in curing wide range of diseases and it is used in unani, siddha and ayurveda treatments[35]. It is an edible fruit widely grown in all over Bangladesh containing the highest source of natural vitamin C[36]. The fruits are highly used in unani medicine to treat diseases like diarrhea, dysentery, diabetes, asthma, bronchitis, cardiac disorder and haemorrhages[37]. It is used for anti-inflammatory[38] and anti-pyretic treatments, and also to treat disorders like scurvy, cancer and heart diseases[39].

The antimicrobial activity of *E. officinalis* extract has been shown in numerous articles against bacterial pathogens but in this study we showed that ethanolic extract of *E. officinalis* is a potent swarming inhibitor of *Proteus* bacteria rather than the antibacterial activity. At the concentration ≥400µg/ml the extract completely inhibited the swarming which is 25 fold lower than that of Cranberry powder. Cranberry powder showed complete inhibition of swarming of *Pm* at 10mg/ml concentration[40]. Moreover, the crude alcoholic extract of *E. officinalis* has been assayed for cellular toxicity to fresh sheep erythrocytes and was found to have no cellular toxicity[15]. Therefore, as a whole our investigation provides information to the physicians that the patients having UTI, CAUTI and would infection may take *E. officinalis* fruit as food supplement along with the prescribed antibiotics to control and manage multidrug resistant *Proteus* bacteria. Twenty phytochemicals are identified so far in fruit pulp of *E. officinalis* including tannins, alkaloids, phenolic compounds, amino acids, carbohydrates, vitamin-C, flavanoids[17]. Therefore, the phytochemical(s) that retarded the swarming of resistant *Proteus* strain is remain to be identified which can be used for the cure and management of recurrent infection caused by *Proteus* and/or other pathogens.

Leaves and fruit pulp of *T. indica* have been extensively investigated but limited information are available on stem bark. Here we found extremely low anti-*Proteus* activity of ethanolic extract of stem bark of *T. indica* but it strongly inhibited the swarming of *Pm*. The MIC of ethanolic extract of *T. indica* bark against *P. mirabilis* has been determined as 20mg/ml by Doughari, 2006[41] which is relatively high concentration. A swarming inhibitor resveratrol, a type of natural phenol produced in several plants, significantly inhibited swarming at 15µg/ml concentration, and completely inhibited swarming at 60µg/ml[34]. Our result made evidence that very low concentration (25µg/ml) of the extract of *T. indica* bark can completely inhibit the active swarming of *Pm* which is comparable to resveratrol. The active component present in that extract should be more potent than resveratrol. However, the inhibitor phytochemical present in *E. officinalis* fruit and *T. indica* bark remain to be identified which might be used as phytomedicine alone or in addition with other antibiotics to control and manage the infection caused by *Proteus* species, and at the same time as a research tool.

Although the molecular mechanisms underlying the inhibition of swarming growth of *Proteus* are not clear, this finding opens up the opportunity to develop drugs that can slowdown the infection caused by *Proteus*, allowing the host enough time to activate defense mechanisms, and to stop and eliminate pathogenic invaders.

Conclusion

The ethanolic extracts of *Emblica officinalis* fruit and *Tamarindus indica* bark potentially inhibited the swarming of pathogenic *Proteus* strain at 400µg/ml and 25µg/ml concentration, respectively. The result has opened up the possibility of the use of these plants in drug development for the treatment of UTI and wound infections. Further investigation is needed for the identification of phytochemical having anti-swarming effect.

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