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Therapeutic Potential of Plant Extracts Against Multidrug Resistance Poultry Bacteria

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Abstract

Plant extracts were evaluated on bacteria isolated from poultry farm for developing substitutive therapeutic agent of antibiotics. A diverse range of bacterial load observed both in total viable count (TVC) and in total coliform count (TCC) in 30 samples randomly collected from poultry feeds, drinking water and faeces. A total of six bacterial isolates e.g. *Pseudomonas* spp., *Aeromonas* spp., *Citrobacter* spp., *Vibrio* spp., *Escherichia coli* and *Plesiomonas* spp. were found in the samples cultured in MacConkey Agar medium. Fifteen antibiotics were studied against bacterial susceptibility. All the bacterial isolates exhibited multi-antibiotic resistance (MAR) with gross resistance to erythromycin and ampicillin. *E. coli* had the highest MAR (53.3%), and *Vibrio* spp. as well as *Plesiomonas* spp. both had the same MAR (46.7%). Methanolic extract of *Terminalia chebula* and *Azadirachta indica* showed significant zone of inhibition against all the tested bacteria. These findings confirm the presence of multidrug resistant bacteria in poultry environment that reveals a possibility of cross-contamination to human and animals. The plant extracts could be developed into therapeutic drugs to rein antibiotic poultry resistant bacteria.

Introduction

Poultry is an agricultural term that refers to all domesticated birds kept for egg-laying and meat production (Danbappa et al. 2018). As a major source of animal protein, human depends largely on poultry (Mulder 1997). It is the second most widely-eaten meat in the world, accounting for about 38% of the world meat (Sule and Ilori 2017). In Bangladesh, a large number of people consume poultry meat and eggs to fulfill their daily protein demand (Rahman et al. 2014). Since early 1990, commercially produced poultry has been

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growing up rapidly in Bangladesh by using improved genetics, manufactured feeds and proper management (Sultana et al. 2017). It was estimated that the poultry meat alone contributes 37% of the total meat production, and about 22-27% of the total animal protein supply in Bangladesh (Hamid et al. 2017). The poultry sector in Bangladesh is expected to employ around 11.2 million people and 2.0 million new households by the year 2020 (Rahman et al. 2017). The poultry industry in Bangladesh is obstructed by a number of constraints of which major one is the outbreak of disease causing mortality of chickens (about 30%) in every year (Sultana et al. 2017). Feed and water are the primary sources of disease causing pathogens in meat and egg producing birds (Adedeji et al. 2015).

Poultry feeds are usually food materials formulated with all nutritional materials (Okonko et al. 2010) needed for proper production of meat and eggs in birds (Chowdhury et al. 2011). Poultry feeds are often contaminated with food borne pathogen during preparation, contaminated raw materials, improper handling, etc. (Chowdhury et al. 2011, Roy et al. 2017). Different bacterial species such as, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Listeria* spp., *Streptococcus* spp., *Klebsiella* spp., *Pseudomonas* spp., etc. found in the poultry feeds could cause diarrhoea, fowl cholera, salmonellosis, staphylococcosis, colibacillosis, erysipelas, listeriosis, etc. in poultry birds (Maciorowski et al. 2007). Water plays an important role in poultry metabolism makes up 55- 75% of the body and elimination of waste products via urine (Jafari et al. 2006). *Campylobacter* spp., *E. coli*, *Pseudomonas* spp. and *Salmonella* spp. are the main poultry pathogens responsible for water contamination along with fecal coliform (Maes et al. 2019).

To improve meat production, the poultry industry uses antibiotics for growth, and disease prevention (Glasgow et al. 2019, Mehdi et al. 2018). Antibiotics have improved poultry performance effectively (Abiala et al. 2016) but in some circumstances pathogenic bacteria like *E. coli*, *Shigella*, *Salmonella*, *Staphylococcus*, *Pseudomonas* showed antibiotic resistance in poultry (Kebede 2010). A large number of antimicrobials used in poultry are also essential for human medicine (Fielding et al. 2012, Agyare et al. 2018). Humans are normally exposed to antimicrobial-resistant bacteria and resistant genes of these microbes are present in human food chain. Therefore, the use of antibiotics must be reduced in poultry industry (Angulo et al. 2009). Instead of antibiotics, plant-based therapeutics can be a good choice for their safety, low toxicity, and environment friendly (Zihadi et al. 2019, Djeussi et al. 2013). The leaf extract of *Azadirachta indica* (local name-Neem) and dried ripe fruit of *Terminalia chebula* (local name-Haritaki) possesses different antimicrobial activities (Kavitha et al. 2017, Ravva and Korn 2015). The leaves of *A. indica* extract showed inhibitory activity against multi-drug resistant human bacterial isolates of *Salmonella typhi, Shigella dysenteriae, E. coli* and *Vibrio cholerae* (Bharitkar et al. 2014) and *T. chebula* extract against *Helicobactor pylori*, *Xanthomonas campestris* and *S. typhi* (Kannan et al. 2009).

Therefore, the aim of this study was to show bacterial association in feeds, water, and chicken poultry faeces, their antibiotics susceptibility and utilization of some plant extracts to minimize the hazards and risks related to bacterial contamination in poultry.

Materials and Methods

A total of 30 samples were collected from poultry practicing rural community in Tangail district of Bangladesh, of which 14 were poultry feed, 9 were water and 7 were faecal samples randomly collected from poultry stores and poultry farms between May and December, 2019. The feed samples were aseptically collected in sterile polyethylene bags while water and faecal materials were aseptically collected in sterile falcon tubes which were sealed and transported to the laboratory directly. A sterile warring blender was used to homogenize 1.0g of each feed and faecal sample into 10 ml of sterile distilled deionized water (Fawole and Oso 2001) resulting 1:10 dilution. Later on, serial dilutions up to 10^{-6} for feed and faecal samples, and 10^{-3} were prepared for water.

For the determination of total viable count (TVC) and total coliform count (TCC), about 0.1ml of diluted samples were cultured in duplicate on nutrient agar and MacConkey agar media, respectively using pour plate method. These were incubated at 37°C for 18hrs in an incubator. The results of TVC were expressed as the number of organism or colony-forming units per gram (cfu/g) of feed and faeces samples and cfu/ml for water sample. The bacterial isolates were identified on the basis of morphological and biochemical tests such as Kligler iron agar (KIA) test, Motility-indole-urease (MIU) test and citrate utilization test (Holt et al. 1994).

The susceptibility of the isolates against some common antibiotics and chemotherapeutics was tested using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar (Hudzicki 2009, Watts et al. 2008). A total of 15 antibiotic discs (Oxoid, UK) such as ampicillin (10 μg), azithromycin (15μg), ciprofloxacin (5μg), tetracycline (30μg), amoxicillin (10μg), nalidixic acid (30μg), and kanamycin (30μg), amikacin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), levofloxacin (5μg), neomycin (30 μg), norfloxacin (10 μg), and tazobactam (110 μg) were used. The interpretation of the antibiotic susceptibility was made according to CLSI (The Clinical and Laboratory Standards Institute 2014).

Healthy and disease-free plant leaves of *Azadirachta indica* were collected directly from the plant and the dried ripe fruit of *Terminalia chebula* purchased from a local market of Tangail. The freshly collected leaves and dried ripe fruits were washed with distilled water and dried in shade for two weeks and blended into powder using mortar. About 100g powder for both *T. chebula* ripe fruit and leaves of *A. indica* were extracted with 600ml of methanol (95% for *T. chebula*), and(30, 40 and 70% methanol for *A. indica*) at 25°C for 48hrs (Sahreen et al. 2011). The crude extracts were filtered using Whatman No. 1 filter paper and evaporated by using a rotary evaporator.

Antimicrobial activity of the plant extracts was tested using disc-diffusion method. Young culture of the test organisms in 2 ml sterile Mueller Hinton broth (MHB) were made from well isolated single colony obtained from 24hrs grown cultures. The test cultures were swabbed on the top of the solidified medium and allowed to dry. The disc containing plant extract (each disc contains 30 µl of plant extract in different methanolic concentration) were placed on the plate. The plates were then incubated at 37°C for 18hrs. The diameter of the zone of inhibition around each disc was measured in mm and the mean value was calculated (Hudzicki 2009).

In this study, microbial were determined following standard formulae. Then the results were analyzed by SPSS ver. 20. Hierarchical analysis was used to estimate overall similarities of the bacterial resistance using their zones of inhibition. Statistical significance was set at a $p<0.05$. Microsoft Excel version 2016 was used to draw graphs wherever appropriate.

Results and Discussion

A diverse range of total viable counts (TVC) and total coliform counts (TCC) of bacteria were found to be associated with the samples (Table1). TVC ranged from 2.64×10^6 to 9.76×10^6cfu/g in poultry feed, 2.6×10^4 to 4.4×10^5 cfu/ml in water and 9.6×10^6 to 1.76×10^8 cfu/g in faeces, respectively. Nasrin et al. (2007) reported that TVC of the faeces, feed and drinking water was $(103.5 \pm 3.62) \times 10^5$, $6.5 \pm 1.87) \times 10^5$ cfu/g and $(31.33 \pm 1.12) \times 10^5$cfu/ml, respectively. Three different samples of poultry faeces contained the most elevated TVC which may be due to the presence of increased number of bacterial populations in gut. On the other hand, TCC for feed, drinking water and faeces of poultry ranged from 1.1×10^5 to 5.84×10^6 cfu/g, 5×10^3 to 5.6×10^4 cfu/ml and 4×10^5 to 1.92×10^7 cfu/g, respectively.

Five bacterial isolates, namely *E. coli*, *Citrobacter* spp., *Pseudomonas* spp., *Aeromonas* spp. and *Vibrio* spp. from poultry feed samples (Layer-layer, Sonali, Layer-starter, Broiler-grower, Broiler-finisher, Broiler-starter), four (*E. coli*, *Citrobacter* spp., *Pseudomonas* spp. and *Plesiomonas* spp.) from drinking water and four (*E. coli*, *Pseudomonas* spp., *Aeromonas* spp. and *Vibrio* spp.) from poultry faeces were found in MacConkey's agar medium (Table 2). Overall, six bacterial isolates were found in feeds, drinking water and faeces of poultry, however, *E. coli* and *Pseudomonas* spp. found in all the three samples. In case of *Pseudomonas* spp. similar result was also reported in feed (Okonko et al. 2010), water (Adesoji et al. 2015) and faeces (Adeleke et al. 2011) of poultry. On the other hand, the other three bacterial isolates e.g. *Citrobacter* spp., *Aeromonas* spp. and *Vibrio* spp. were found in any two samples, but *Plesiomonas* spp. found only in water (Table 2).

Biochemical tests, alternatively, confirm further the mentioned isolates of all bacteria found in three samples (Table 3). This characterization was accomplished simultaneously by observing distinct morphological characteristics and a number of biochemical tests on the basis of presence (+) or absence (-) criterion in Tables 2 and 3.

Table 1. Total viable counts (TVC) and total coliform counts (TCC)of the feed, water and poultry faeces.

Sample	TVC (cfu/g)or (cfu/ml)	TCC (cfu/g) or (cfu/ml)
Feed-1 (Layer-layer)	7.20×10^6	1.4×10^6
Feed-2 (Layer-layer)	4.35×10^6	3.60×10^6
Feed-3 (Sonali)	4.20×10^6	1.85×10^6
Feed-4 (Sonali)	3.52×10^6	2.60×10^6
Feed-5 (Layer-starter)	3.40×10^6	2.12×10^6
Feed-6 (Layer-layer)	3.16×10^6	2.05×10^6
Feed-7 (Layer-layer)	2.76×10^6	1.52×10^6
Feed-8 (Broiler-finisher)	3.84×10^6	4.8×10^5
Feed-9 (Broiler-finisher)	5.60×10^6	2.56×10^6
Feed-10 (Broiler-grower)	2.64×10^6	1.1×10^5
Feed-11 (Broiler-grower)	5.6×10^6	4.56×10^6
Feed-12 (Broiler-grower)	8.96×10^6	3.28×10^6
Feed-13 (Layer-layer)	9.76×10^6	5.84×10^6
Feed-14 (Broiler-starter)	5.44×10^6	8.2×10^5
Water-1	1.72×10^5	2.0×10^4
Water-2	1.48×10^5	2.8×10^4
Water-3	4.4×10^5	1.6×10^4
Water-4	9.2×10^4	5.6×10^4
Water-5	1.18×10^5	1.7×10^4
Water-6	9×10^4	2.3×10^4
Water-7	1.2×10^5	4.5×10^4
Water-8	3.1×10^4	5×10^3
Water-9	2.6×10^4	7×10^3
Faeces-1	1.2×10^8	4×10^5
Faeces-2	1.76×10^8	8.4×10^6
Faeces-3	9.60×10^7	1.92×10^7
Faeces-4	1.14×10^8	1.15×10^7
Faeces-5	1.04×10^7	8.4×10^6
Faeces-6	2.56×10^7	9×10^5
Faeces-7	9.6×10^6	6.0×10^6

Table 2. The bacteria found in feeds, water and poultry faeces in MacConkey's agar medium.

Isolates	Feed	Drinking water	Faeces
Escherichia coli	+	+	+
Citrobacter spp.	+	+	-
Pseudomonas spp.	+	+	+
Aeromonas spp.	+	-	+
Plesiomonas spp.	-	+	-
Vibrio spp.	+	-	+

+ = Presence, – = Absence.

Table 3. Major biochemical tests for the bacteria isolated from feeds, drinking water and poultry faeces growing on MacConkey agar medium.

Biochemical tests								Presumptive identified organisms
KIA				MIU			Simon's Citrate	
Slant	Butt	Gas	H_2S	Motility	Indole	Urease		
K	A	+	-	+	+	-	+	*Citrobacter* spp.
A	A	+	-	+	+	-	-	*Escherichia coli*
K	K	-	-	+	-	+	+	*Pseudomonas* spp.
K	A	+	-	+	+	-	+	*Aeromonas* spp.
K	K	-	-	+	+	-	+	*Plesiomona* spp.
K	A	-	-	+	+	-	+	*Vibrio* spp.

K = Alkaline, A = Acidic, '+' = Presence, '–' = Absence.

Six bacterial isolates *viz., Pseudomonas* spp., *Aeromonas* spp., *Citrobacter* spp., *Vibrio* spp., *E. coli* and *Plesiomonas* spp. isolated from poultry chicken sources were used to test antimicrobial susceptibility against 15 commercial antibiotics. Percentage of resistance observed from the isolates were 26.7, 40, 26.7, 46.7, 53.3 and 46.7% against *Pseudomonas* spp., *Aeromonas* spp., *Citrobacter* spp., *Vibrio* spp., *E. coli* and *Plesiomonas* spp., respectively (Table 4). All the bacterial isolates showed gross resistance against erythromycin, ampicillin and gross susceptible against tazobactam, gentamicin, ciprofloxacin, amikacin, chloramphenicol (Table 4).

In this study, presence of *E. coli* in the feed, drinking water and faeces of poultry chickens was found resistant to multiple antibiotics. The pathogenic strains did not only increase the resistance against the antibiotics but also increased resistance in the endogenous flora of humans and animals (Kolář et al. 2002). *E. coli* isolates showed resistance against amoxicillin, tetracycline, erythromycin, norfloxacin, levofloxacin, ampicillin, azithromycin and nalidixic acid. Chowdhury et al. (2011) reported that *E. coli* isolates from poultry in Savar of Bangladesh were found resistant to chloramphenicol,

ampicillin, ciprofloxacin and tetracycline. It is not clear as to why this discrepancy was found in case of ciprofloxacin and chloramphenicol. It could be due to different strains/serotypes obtained from different locations.

Table 4. Antibiotic susceptibility profiles of the bacteria isolated from different poultry samples.

Antibiotics	Code	Potency (µg)	*Pseudomonas* spp. (%)	*Aeromonas* spp. (%)	*Citrobacter* spp. (%)	*Vibrio* spp. (%)	*E. coli* (%)	*Plesiomonas* spp. (%)
Amoxicillin	AX	10	S	R	R	R	R	R
Tetracycline	TE	30	S	S	S	R	R	R
Erythromycin	E	15	R	R	R	R	R	R
Neomycin	N	30	R	R	S	R	S	S
Norfloxacin	NOR	10	S	S	S	S	R	S
Tazobactam	TPZ	110	S	S	S	S	S	S
Kanamycin	K	30	S	S	S	R	S	R
Gentamicin	CN	10	S	S	S	S	S	S
Levofloxacin	LEV	5	R	S	S	S	R	S
Ciprofloxacin	CIP	5	S	S	S	S	S	S
Amikacin	AK	30	S	S	S	S	S	S
Ampicillin	AM	10	R	R	R	R	R	R
Azithromycin	AZM	15	S	R	R	R	R	R
Nalidixic Acid	NA	30	S	R	S	S	R	R
Chloramphenicol	C	30	S	S	S	S	S	S
MAR (%)			26.7	40.0	26.7	46.7	53.3	46.7

R= Resistance, S= Susceptible.

Pseudomonas was one of the most frequently identified bacteria associated with all samples exhibited resistance to erythromycin, neomycin, ampicillin, and levofloxacin. With reference to Nigeria, *Pseudomonas* found resistant to all their test antibiotics except gentamicin (Okonko et al. 2010). Our findings showed similar consequence in regard to gentamicin also. Surprisingly, *Pseudomonas* spp. showed sensitivity against amoxicillin and azithromycin compared to other isolates which were found resistant to them.

The presence of *Vibrio* spp. in feed (Roy et al. 2017) and *Plesiomonas* spp. in water (Pilar and De Garcia 1997, Santos et al. 2015) has been re-established by our findings. The isolated *Vibrio* sp. and *Plesiomonas* spp. were found resistant to amoxicillin, tetracycline, erythromycin, kanamycin, ampicillin, and azithromycin. The observed resistance against these antibiotics that were used presumably enlightened the high usage of the drugs in the study sites. As a result, these drugs may have become seriously imperiled and ineffective. *Vibrio* spp. and *Plesiomonas* spp. displayed resistance to tetracycline and kanamycin and they both had the same MAR (46.7%).

Aeromonas ssp. and *Citrobacter* spp. both were found in feed and faeces of poultry. These bacteria were resistant to amoxicillin, erythromycin, ampicillin, azithromycin. In another study, *Aeromonas* spp. was found resistant to erythromycin (Igbinosa 2014) and *Citrobacter* spp. showed resistance profile against tetracycline, gentamicin, nalidixic acid and chloramphenicol (Kannan et al. 2009).

The hierarchical analysis of the Gram negative bacteria isolates conjectured that the antibiotic resistance pattern of *Vibrio* spp. (4) and *Plesiomonas* spp.(6); *Pseudomonas* spp. and *Citrobacter* spp. were the most related. The other isolates, found from poultry environment were less related (Fig. 1).

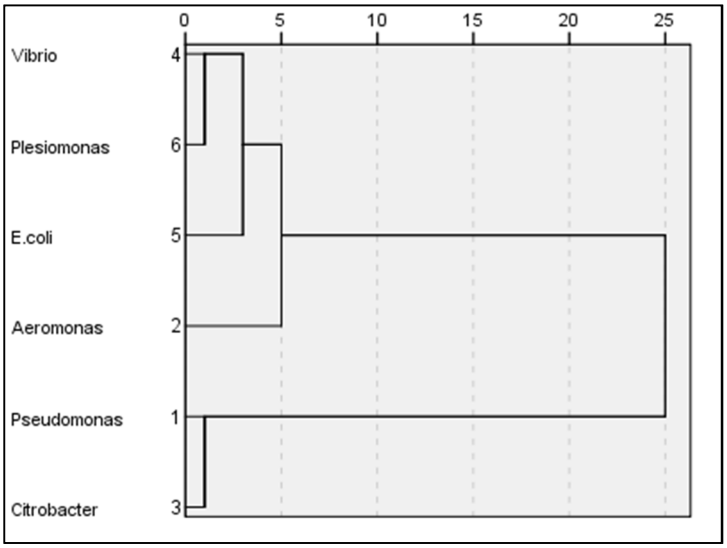

Fig. 1. Hierarchical analysis of antibiotic resistance pattern of the Gram negative bacteria isolated from poultry environment. 1 = *Pseudomonas* spp., 2 = *Aeromonas* spp., 3 = *Citrobacter* spp., 4 = *Vibrio* spp., 5 = *E. coli* and 6 = *Plesiomonas* spp.

Antibacterial activities of both 95% methanolic *Terminalia chebula* extract and 70% methanolic *Azadirachta indica* extract were significant against all the isolated bacteria that were resistant against different antibiotics. The average zone of inhibition of 95% methanolic *Terminalia chebula* extract and 70% methanolic *Azadirachta indica* extract were 11.33±1.25 and 12.33±1.25, respectively. On the other hand, 30 and 40% methanolic extract of *Azadirachta indica* have less significant zone of inhibition against the tested bacteria (Table 5).

The methanolic extract of *T. chebula* and *A. indica* extracts demonstrated antibacterial activities against antibiotic resistant *E. coli*. This observation is in conformity with other workers (Mostafa et al. 2011, El-Moez et al. 2014). The activity of *T. chebula* (Kannan et al. 2009) and *A. indica* (Harjai et al. 2013) was evident against *Pseudomonas.* The antibacterial

activity of plant extracts inhibits the growth of *Vibrio* and *Plesiomonas*. This finding was consistent with previous reports (Mostafa et al. 2011, Thakurta et al. 2007) by using *T. chebula* and *A. indica* extract against *Vibrio*. However, *Aeromonas* and *Citrobacter* spp. both have the susceptibility against the *A. indica* extract (El-Moez et al. 2014, Dhayanithi et al. 2010) as well as *T. chebula* extract.

Table 5. Antibacterial activity of *Terminalia chebula* and *Azadirachta indica* plant extracts.

Isolates	Inhibition zone (mm)			
	95% methanolic *T. chebula* extract	70% methanolic *A. indica* extract	40% methanolic *A. indica* extract	30% methanolic *A. indica* extract
Pseudomonas spp.	11	13	0	0
Aeromonas spp.	13	12	0	0
Citrobacter spp.	12	13	0	0
Vibrio spp.	11	12	0	0
E. coli	9	10	0	0
Plesiomonas spp.	12	14	0	0
Mean ± SD	11.33± 1.25	12.33± 1.25	0	0

There was no significant relationship between *Terminalia chebula* and *Azadirachta indica* extract against the six bacteria isolated from different poultry sources (t value = 1.85, p 0.138) (Table 6).

Table 6. Statistical t value, p value and 95% confidence interval of *T. chebula* and *A. indica* extract on bacteria isolated from different poultry samples.

R	df	F	T value	95% confidence interval		p value
				Lower	Upper	
0.679	1	3.41	1.85	–0.341	1.7	0.138

Poultry environment serves as a source of multidrug resistance bacteria. These bacteria not only infect the poultry but also can infect or reach into the human population through farm to fork model. The indiscriminate and overuse of antibiotics as growth promoters and prevention of diseases are behind the incidence of resistance. Therefore, an alternative approach is important to reveal the plants extract which contains the bioactive compounds. Thus, an onward action plan might be taken nationwide to monitor the use of antibiotics and the application of therapeutic use of medicinal plants will ultimately reduce resistant bacteria.

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