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Extract of neem (*Azadirachta indica*) leaf exhibits bactericidal effect against multidrug resistant pathogenic bacteria of poultry

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Abstract

The aim of the study was to determine the efficacy of neem leaf extract against multidrug resistant (MDR) pathogenic bacteria. Laboratory stock culture of *Pasteurella multocida*, *Salmonella pullorum*, *Salmonella gallinarum* and *Escherichia coli* was revived. Antibiogram profiles of these bacteria were determined by disc diffusion method. Ethanolic extract of neem leaf was prepared. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of neem leaf extract (112.5, 100, 50, 25, 12.5, 6.25 and 3.12 mg/ml) against MDR pathogenic bacteria of poultry were determined by double dilution method. The MIC and MBC of the neem leaf extract were 12.5 and 25 mg/ml, respectively for *P. multocida*, 50 and 100 mg/ml for *S. pullorum* and *S. gallinarum*, 100 and 112.5 mg/ml for *E. coli*. Neem leaf extracts exhibited bactericidal effect against MDR pathogenic bacteria of poultry.

KEYWORDS
antibacterial efficacy, multidrug resistant pathogenic bacteria, neem leaf extract, poultry

1 | INTRODUCTION

Antibiotics are medicines that either kill or inhibit the growth of bacteria. In poultry, husbandry antibiotics are frequently used for prevention and control of bacterial infections. Long-term and indiscriminate use of antibiotics leads to the development of antibiotic resistance in bacteria (Diarra & Malouin, 2014; Forgetta et al., 2012; Furtula et al., 2010) and reduce the number of beneficial gut microbiota in poultry (Yadav & Jha, 2019). Excessive use of antibiotics results in residues in meat and egg of poultry which affect consumer's health (Mehdi et al., 2018). Hence, there is an urgent need to find out suitable alternatives of antibiotics to control bacterial infections in order to stop the emergence of antibiotic resistant bacteria in poultry.

Medicinal plants known to contain antimicrobial compounds (Frankic et al., 2009; Ghasemi et al., 2014; Mehdi et al., 2018; Toghyani et al., 2011; Windisch et al., 2008). A good number of plants possess therapeutic properties against bacterial infections (Kayode & Kayode, 2011). The neem (*Azadirachta indica*) is one of the popular medicinal plants in the South East Asia (Murthy & Sexena, 1998). It is also found in many countries of the world having tropical and subtropical climates (Alzohairy, 2016). It is very often used in Ayurveda, Unani and Homoeopathic medicines for its antimicrobial properties (Lakshmi et al., 2015). There are more than 140 bioactive compounds found in neem (Subapriya & Nagini, 2005). Azadirachtin, nimbin and nimbidine are the most abundance bioactive compounds found in the leaves of Neem (Mondali et al., 2009). The leaves, flowers, seeds, fruits, roots and bark of neem tree are used for the treatment

of infections on skin, teeth and gums (Subapriya & Nagini, 2005). The leaves of neem are used to treat skin allergies, and healing of wound of small pox and chicken pox (Hla et al., 2011). The antimicrobial activity of neem leaves extract against *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., *E. coli*, and some fungal strains have been reported (Koona & Budida, 2011; Valarmathy et al., 2010). The use of neem leaf extract in immunosuppressed birds increased humoral and cell mediate immunity (Sadekar et al., 1998).

Pasteurella multocida, *Salmonella pullorum*, *Salmonella gallinarum* and *Escherichia coli* are known to cause fowl cholera, fowl typhoid, pullorum diseases, and colibacillosis in poultry. These bacterial diseases are prevalent in Bangladesh and causing significant economic losses in poultry industries due to high morbidity and mortality. Antibiotics are being used for treating these bacterial diseases which often leads to the developments of multidrug resistant (MDR) bacteria. Extract of neem leaf is known to have antibacterial activity (Akhter & Sarker, 2019) without drug resistant problem. Therefore neem leaf extract may be used as an alternative to antibiotics to treat MDR bacterial diseases. The objective of the present research was to determine antibacterial efficacy of neem leaf extract against MDR pathogenic bacteria of poultry: *P. multocida*, *S. pullorum*, *S. gallinarum* and *E. coli*.

2 | MATERIALS AND METHODS

2.1 | Collection of neem leaf

The neem leaves were collected from Jahangir Nagar University campus, Savar, Dhaka. The collected samples were packed in plastic containers and were transported to the Department of Pharmacy at the Jahangir Nagar University, Savar, Dhaka, for preparation of neem leaf extract.

2.2 | Preparation of neem leaf extract

Neem leaves (500 g) were thoroughly washed in water and dried at 35°C–40°C and pulverized in an electric grinder. Pulverized neem leaf was taken in soxhlet apparatus with 3,000 ml of 96% ethanol and heated at 78°C for 18 hr. Then, the ethanolic extract of neem leaf powder was dried in a Rota Vapor (BUCHI Rota Vapor R-114, Switzerland) to get a solid mass which was stored at 4°C until use.

2.3 | Bacterial strains

Laboratory stock of pathogenic bacteria of poultry such as *P. multocida*, *S. pullorum*, *S. gallinarum*, and *E. coli* were revived by culturing onto Blood agar (*P. multocida*), Salmonella Shigella (SS) agar (*S. pullorum and S. gallinarum*) and Eosine Methylene Blue (EMB) agar (*E. coli*). *P. multocida*, *S. pullorum*, *S. gallinarum*, and *E. coli* were previously isolated from poultry and identified by cultural characteristics; biochemical tests and PCR assays (Matin et al., 2017; Panna et al., 2015; Parvej et al., 2016).

2.4 | Antibiotic susceptibility test

Antibiotic susceptibility profiles of bacteria were tested by disc diffusion method (Baurer et al., 1966) against 14 commercially available antibiotics such as Ampicillin, Gentamycin, Azithromycin, Sulphatrimethoprim, Vancomycin, Ciprofloxacin, Cephalexin, Doxycycline, Streptomycin, Oxacillin, Erythromycin, Tetracycline, Chloramphenicol and Nalidixic acid (Hi Media, India). Antimicrobial testing results were recorded as susceptible, intermediate and resistant according to zone diameter interpretive standards provided by CLSI (2010).

2.5 | Determination of minimum inhibitory concentration and minimum bactericidal concentration

The MIC and MBC of neem leaf extract was determined by doubling dilution method (Sahm and Washington, 1990). Briefly, 1-ml neem leaf extract (200 mg/ml) was placed in 1-ml Muller Hilton broth; 1 ml of this extract concentration was transferred to another test tube and this dilution continued until concentrations of 100, 50, 25, 12.5, 6.25 and 3,125 mg/ml reached in different test tubes; 112.5 mg/ml concentration of neef leaf extract was prepared separately by placing 1-ml neef leaf extract (225 mg/ml) into 1-ml Muller Hilton broth. One test tube was filled with 1ml of Muller Hilton broth (negative control), and four test tubes were kept for positive control using ciprofloxacin (1 μg/ml for *P. multocida* and 8 μg for *S. pullorum*, *S. gallinarum* and *E. coli*). Twenty microliter 0.5 McFarland turbidity standard bacterial suspension (109 cfu/ml) was added into the all concentration in test tubes and incubated at 37°C for 24 hr. Four separate experiments were conducted for four bacteria such as *P. multocida*, *S. pullorum*, *S. gallinarum* and *E. coli*. Bactericidal activity of neem leaf extract at different concentration (100, 50, 25, 12.5, 6.25 and 3.125 mg/ml) was determined by measuring colony forming unit (CFU)/ml of bacteria after incubation. Briefly, Muller Hilton broth (450 μl) was taken into a series of eppendorf tubes. Overnight treated culture (50 μl) was added into the first eppendorf tube and mixed well by vortexing, and then tenfold serial dilution ranging from 10^{-1} to 10^{-10} was prepared. Fifty microliter suspension from 10^{-4} to 10^{-10} was plated duplicate onto Blood agar (for *P. multocida)* SS agar (for *S. pullorum* and *S. gallinarum)* EMB agar (for *E. coli)*. Inoculated plates were incubated at 37°C for 24 hr. Numbers of colonies on the plates were counted and CFU/ml was measured using following formula:CFU/ml = Number of colony × Dilution factor. Turbidity of the neem leaves extract at different concentrations was measured immediately after adding 20 μl of 0.5 McFarland turbidity standard bacteria into each dilution by a spectrophotometer at 580 nm wavelength. Turbidity

was measured again after 24 hr of incubation. The minimum concentration of neem leaf extract that inhibit the growth of bacteria was considered as MIC of the extract (Hugo and Russel, 1983). The minimum concentration of the extract that completely inhibits the growth of bacteria was considered as minimum bactericidal concentration (MBC) (De & Ifeoma, 2002).

2.6 | Statistical analysis

Efficacy of different concentrations of neem leaf extract was compared for statistical significance using student *t* test with SPSS. A '*p*' value of ≤0.05 was considered as statistically significant.

3 | RESULTS

3.1 | Cultural characteristics of bacteria

On blood agar *P. multocida* exhibited grayish circular non hemolytic colonies (Figure 1a). Both *S. gallinarum* and *S. pullorum* produced circular opaque black colonies (Figure 1b,c) and *E. coli* on EMB agar produced greenish colonies with metallic sheen (Figure 1d).

3.2 | MDR profiles of poultry bacteria

The highest number of antibiotics resistant was recorded for *E. coli* (resistant to 5 classes of antibiotics) followed by *S. gallinarum* and *S. pullorum* (resistant to 4 classes of antibiotics) and *P. multocida* (resistant to 3 classes of antibiotics) (Table 1).

3.3 | The MIC and MBC of neem leaf extract against P. multocida

The neem leaves extract at 100 mg/ml, 50 mg/ml and 25mg/ml concentrations completely inhibited the growth of *P. multocida* which were statistically significant ($p < .001$). At the concentration of 12.5 mg/ml, it significantly reduced the growth of bacteria (log 7.98 ± 0.52 CFU/ml) as compared to untreated control (log 10.98 ± 0.47 CFU/ml) ($p < .001$). The growth of *P. multocida* was not inhibited at 6.25 and 3.125 mg/ml concentrations. The MIC and MBC of neem leaf extract against *P. multocida* was recorded as 12.5 mg/ml and 25 mg/ml, respectively (Table 2).

3.4 | The MIC and MBC of neem leaf extract against S. pullorum

A complete growth inhibition of *S. pullorum* was recorded at 100mg/ml concentration of neem leaf extract ($p < .001$). At 50 mg/ml concentration, it significantly reduced the growth of bacteria (log 8.02 ± 0.41 CFU/ml) as compared to untreated control (log 12.29 ± 0.25 CFU/ml) ($p < .001$). No growth inhibition of *S. pullorum* was seen both at 6.25 and 3.125 mg/ml concentrations. The MIC and MBC of neem leaf extract against *S. pullorum* were 50 mg/ml and 100 mg/ml, respectively (Table 3).

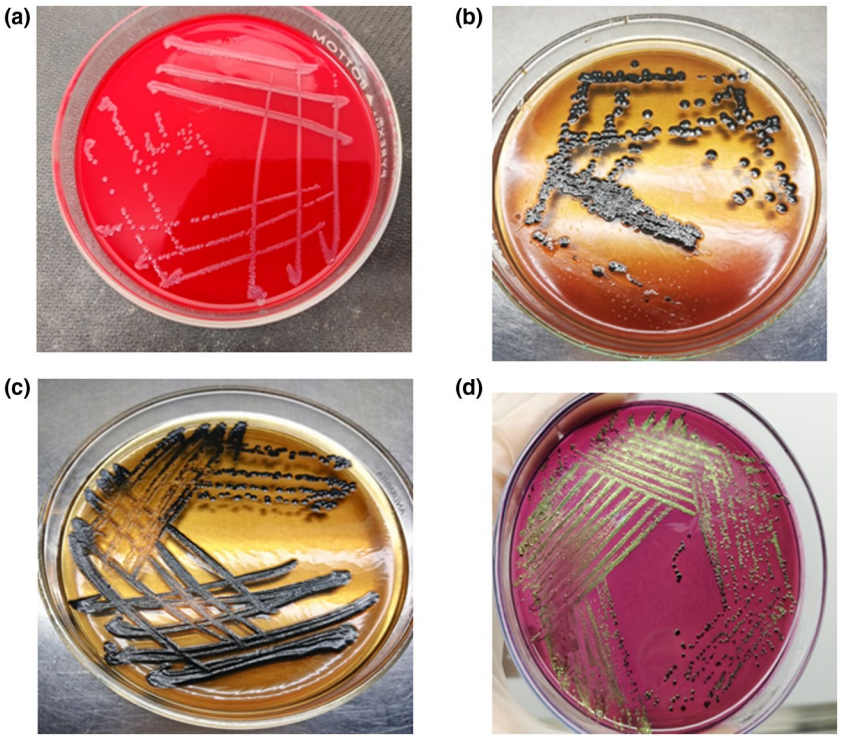

FIGURE 1 Cultural characteristics of bacteria used in this study. Grayish circular and nonhemolytic colonies of *P. multocida* on blood agar (a), circular black colonies of *S. pullorum* (b) and *S. gallinarum* on Salmonella-Shigella agar (c), greenish colored metallic sheen colonies of *E. coli* on Eosine Methylene Blue agar (d)

TABLE 1 Antimicrobial classes and antimicrobials used to determine multidrug resistant profiles of bacteria isolated from poultry

Name of bacteria	Antimicrobial classes	Antimicrobial agents	Results of antimicrobial susceptibility testing (S/I/R)
Pasteurella multocida	Aminoglycoside	Streptomycin	R
	Sulphonamides and Trimethoprim	Sulphatrimethoprim	
	Quinolone	Nalidixic acid	
Salmonella pullorum	Cephalosporin	Cephalexin	R
	Glycopeptide	Vancomycin	
	Aminoglycoside	Streptomycin	
	Macrolide	Erythromycin	
Salmonella gallinarum	Cephalosporin	Cephalexin	R
	Glycopeptide	Vancomycin	
	Aminoglycoside	Streptomycin	
	Macrolide	Erythromycin	
Escherichia coli	Aminoglycoside	Streptomycin	R
	Macrolide	Azithromycin	
	Cephalosporin	Cephalexin	
	Glycopeptide	Vancomycin	
	Tetracycline	Doxycillin	

Abbreviations: I, intermediate; R, resistant; S, susceptible.

TABLE 2 Minimum growth inhibitory and bactericidal concentration of ethanolic extract of neem leaf against *Pasteurella multocida*

Concentration (mg/ml)	Log CFU/ml	Log reduction
0	10.98 ± 0.47	0
3.125	10.98 ± 0.35	0.004
6.25	10.96 ± 0.36	0.027
12.5[a]	7.98 ± 0.52	3.05
25[b]	0	10.98
50	0	10.98
100	0	10.98

[a]Minimum inhibitory concentration.
[b]Minimum bactericidal concentration.

TABLE 3 Minimum growth inhibitory and bactericidal concentration of ethanolic extract of neem leaf against *Salmonella pullorum*

Concentration (mg/ml)	Log CFU/ml	Log reduction
0	12.29 ± 0.25	0
3.125	12.29 ± 0.77	0
6.25	12.28 ± 0.23	0.1
12.5	10.31 ± 0.28	1.99
25	9.4 ± 0.13	2.9
50[a]	8.02 ± 0.41	4.24
100[b]	0	12.29

[a]Minimum inhibitory concentration.
[b]Minimum bactericidal concentration.

3.5 | The MIC and MBC of neem leaf extract against S. gallinarum

Neem leaf extract at 100 mg/ml concentration completely inhibited the growth of *S. gallinarum* ($p < .001$). At 50 mg/ml concentration, the neem leaf extract significantly reduced the growth of bacteria (log 8.02 ± 0.41 CFU/ml) when compared to untreated control (log 12.29 ± 0.25 CFU/ml) ($p < .001$). At 6.25 and 3.125 mg/ml concentrations, these did not exhibit growth inhibitory effect against *S. gallinarum*. The MIC and MBC of neem leaves extract against *S. gallinarum* were 50 mg/ml and 100 mg/ml, respectively (Table 4).

3.6 | The MIC and MBC of neem leaf extract against E. coli

The concentration of ethanolic extract at 112.5 mg/ml completely inhibited the growth of *E. coli* and it was statistically significant ($p < .001$). At 100 mg/ml concentration a significant growth reduction of *E. coli* (log 8.03 ± 0.59 CFU/ml) was found as compared to untreated control (log 13.22 ± 0.27 CFU/ml) ($p < .001$). At 6.25 and 3.125 mg/ml, concentrations neem leaf extract did not exhibit the growth inhibitory effect against *E coli*. The MIC and MBC of neem leaves extract against *E. coli* were 100 mg/ml and 112.5 mg/ml, respectively (Table 5).

TABLE 4 Minimum growth inhibitory and bactericidal concentration of ethanolic extract of neem leaf against *Salmonella gallinarum*

Concentration (mg/ml)	Log CFU/ml	Log reduction
0	12.29 ± 0.25	0
3.125	12.29 ± 0.77	0
6.25	12.28 ± 0.23	0.1
12.5	10.31 ± 0.28	1.99
25	9.4 ± 0.13	2.9
50[a]	8.02 ± 0.41	4.24
100[b]	0	12.29

[a]Minimum inhibitory concentration.

[b]Minimum bactericidal concentration.

TABLE 5 Minimum growth inhibitory and bactericidal concentration of ethanolic extract of neem leaf against *Escherichia coli*

Concentration (mg/ml)	Log CFU/ml	Log reduction
0	13.22 ± 0.27	0
6.25	13.21 ± 0.25	0.003
12.5	13.19 ± 0.19	0.2
25	11.2 ± 0.27	2.02
50	9.21 ± 0.21	4.02
100[a]	8.03 ± 0.59	5.15
112.5[b]	0	13.22

[a]Minimum inhibitory concentration.

[b]Minimum bactericidal concentration.

4 | DISCUSSION

Antibiotics are widely used in poultry husbandry as a growth promoter as well as for prophylaxis and therapeutic purpose against bacterial diseases (Diarra & Malouin, 2014). Inappropriate and excessive uses of antibiotics are responsible for the development of multidrug resistant (MDR) bacteria in poultry and its farm environments. The multidrug resistant genes of bacteria can spread rapidly among bacterial population (Szmolka & Nagy, 2013). Residual antibiotics present in the meat and eggs may lead to the development of drug resistant bacteria in humans (Diarra & Malouin, 2014). Alternative therapies other than antibiotics need to be developed to stop emergence of MDR bacteria in humans and animals. The medicinal plants are known to contain bioactive compounds which are widely used for treatment of bacterial infections (Al-Hashemi & Hossain, 2016). Antimicrobial properties of neem leaf have been well established. Therefore, the present study was carried out to test the efficacy of neem leaf extract against MDR bacteria of poultry.

In the present study antibiogram profile of four pathogenic bacteria of poultry showed that they were resistant to at least three different classes of antibiotics. It indicates that they were MDR bacteria. Although this study detected only phenotypic resistant profiles of bacteria by disc diffusion method but simultaneous detection of drug resistant genes by PCR could help additional confirmation of this resistant phenotype. The MDR bacteria were reported in poultry and its environments (Khatun et al., 2016; Parveen et al., 2007; Reza et al., 2015). Bacteria present in poultry and its farm environment are continuously exposed to antibiotics which favor the development of MDR. Infections caused by MDR bacteria in humans and animals are very difficult to treat. Bioactive compounds in neem leaf extract could be the inexpensive antimicrobial agents with high safety and efficacy (Reddy et al., 2013). Neem leaf extract showed potential antibacterial activity (Koona & Budida, 2011). The neem oil was also found to be effective in killing multidrug resistant bacteria isolated from humans (Jain et al., 2013). A water-soluble glycolipid, sulfonoquinovosyldiacylglyceride, isolated from the leaves of neem showed inhibitory activity against *Salmonella typhi*, *Shigella dysenteriae*, *E. coli*, and *Vibrio cholera* (Bharitkar et al., 2014). In this study, the MDR bacteria resistant to more number of antibiotics required higher concentration of neem leaf extract compared to MDR bacteria resistant to lower number of antibiotics for complete growth inhibition. In the present study, the MIC of neem leaf extract was higher for *E. coli* compared to *P. multocida*, *S. pullorum* and *S. gallinarum*. The efficacy of neem leaf extract as well as neem oil were found to be less effective against *E. coli* (Koona & Budida, 2011; SaiRam et al., 2000). The MIC of neem leaf extract was ranged between of 500 and 2000 μg/ml for bacteria (*Enterococcus feacalis*, *Staphylococcus aureus*, *Pseudomonas aeuroginosa* and *Proteus mirabilis*) and fungi (*Aspergillus fumigatus* and *Candida albicans*) by agar well diffusion method (Reddy et al., 2013).

5 | CONCLUSION

In this study, the ethanolic extract of neem leaf exhibited antibacterial activity against the MDR bacteria of poultry. Further in vivo study need to be carried out in poultry to observe its antibacterial efficacy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Edris Ali: Conceptualization; Investigation; Writing-original draft; Writing-review & editing. **Md. Sadequl Islam:** Conceptualization; Methodology; Writing-original draft; Writing-review & editing. **Md. Ismail Hossen:** Methodology; Writing-review & editing. **Mst. Minara Khatun:** Data curation; Investigation; Methodology; Writing-review & editing. **Md. Ariful Islam:** Conceptualization; Data curation; Investigation; Supervision; Writing-original draft; Writing-review & editing.

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