Antibacterial efficacy of *Lawsonia inermis* against *Escherichia coli* of commercial broiler chicken

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Chittagong Veterinary and Animal Sciences University

Zakir Hossain Road, Khulshi, Chittagong-4225

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Clinical Report Submitted as per approved style and content

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ABSTRACT

Globe is gifted with worthy wealth of medicinal plant by almighty. The plant kingdom played an important role in survival of man by providing their products and active constituent. There is a far-reaching reliance that green medicines are healthier and more in noxious or more secured than synthetic one. The use of medicinal plant in pharmaceutical industry is going to be more popular due to inability to cause any side effect to patient and not creating any resistance to pathogenic organism where as many antibiotic are resistant to some harmful organism. *Escherichia coli* is a harmful Gram negative organism that cause colibacillosis in chicken and cause heavy economic losses in poultry farm. After collection of leave of *Lawsonia inermis* (henna) drying is done to make powder and then methanol as solvent is added to grinded leave. Finally extract was got by evaporating the methanol by rotary evaporator. Different concentrations were made by disk diffusion method the study was carried out. The methanolic extract of *Lawsonia inermis* leave showed a moderate sensitivity to this organism at 100mg/µL and create zone of inhibition measured 18mm in diameter. This medicinal plant has the antibacterial activities and act well against some bacteria like *E coli*. After doing experiment it can be recommended that the extract of henna can be used at certain concentration as a remedy of antibiotic resistance.

Keywords: Medicinal plant, resistance*, Escherichia coli, Lawsonia inermis*, inhibition

CHAPTER: I
INTRODUCTION

According to World Health Organization (WHO) medicinal plants would be the best natural source to obtain a variety of drugs. In developed countries about 80% individual use traditional medicines, this has compounds derived from various medicinal plants (Ellof, 1998). Medicinal plants synthesize secondary metabolites which have antimicrobial activity and thus in nowadays the use of these plants get popular. The higher plants represent a potential source of novel antibiotic prototypes which is found after screening of these plants for antimicrobial activity (Selvamohan *et al*., 2012). In recent years, indiscriminate use of commercial antimicrobial drug in many infectious diseases produce increased microbial resistance to many pathogenic organisms in animal. Thus the scientists have been forced to search new antimicrobials from different sources like medicinal plants (Wu *et al*., 1999). Plant produces a wide variety of secondary metabolites which has either direct use as precursors or as lead compounds in the pharmaceutical industry. It is expected that plant extracts showing target sites in a better way other than those used by antibiotics will be active against drug resistant pathogenic microbes. However, very little information is available on such activity of medicinal plants, and out of the 400,000 plant species in globe, only a smaller portion has been investigated systematically for their antimicrobial activities (Anjana *et al*., 2009). Medicinal herbs possess curative properties Due to the presence of various complex chemical substance of different composition, medicinal herbs have curative properties and the substances are found as secondary plant metabolites in one or more parts of these plants (Patil *et al*., 2009). In this context, the status of antimicrobial activity of widely used traditional medicinal plants in Bangladesh, *Lawsonia inermis* Linn. Lythraceae which have been used for several purposes including treatment of various diseases has studied.

It is a biennial dicotyledonous herbaceous shrub. It is a native of North Africa and South-West Asia. As an ornamental and dye plant, nowadays the plant is widely cultivated throughout the tropics. Quadrangular branches are green in color in early stage and which turn red with age. In early stage the bark is grayish brown and unarmed but branches are spine tipped in later stage. Flowers with four crumbled petals are small, numerous, fragrant, white or rose colored. Fruit looks like small capsule which is brown in color. When fruits get ripened, they open irregularly

and split into four compartment and is many seeded. Seeds are about 3 mm across, numerous, smooth, pyramidal, hard and thick seed coat with brownish coloration. Henna has been used as cosmetic and medicine for over 9,000 years. Traditionally in Bangladesh, henna is applied to hands and feet. In India henna became very popular for its cooling effect in the hot summer. Henna leaves, flowers, seeds, stem bark and roots are used in traditional medicine to treat a variety of ailments such as headache, ulcers, diarrhea, leprosy, fever, leucorrhoea, diabetes, rheumatoid arthritis cardiac disease, hepatoprotective and coloring agent (Choudhary *et al*., 2010). The main uses of henna are as a cooling agent, astringent, anti-fungal and anti-bacterial herb for the hair and skin. Its core chemical components are 2- hydroxynapthoquinone (lawsone), mannite, tannic acid, mucilage and gallic acid. Out of these the main one is 2-hydroxynaphthoquinone. About 0.5-1.5% of henna is made of lawsone. Its bioactive feature is thought to be due to its high protein binding capacity. Previously many researchers experimented about the antimicrobial activity of henna in human against some pathogenic microorganism like *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Bacillus anthracis etc.*

To meet up the increasing demand of animal protein,poultry farming is going to be more popular among rural peoples, traders and other commercial enterprise. Poultry farming is not only for production of chicken, egg but also it creates huge employment opportunity to the overgrowing population of Bangladesh. Thus poultry farming plays an important role in alleviating the poverty and in income generation in our country. But diseases of poultry are the main constraint to the poultry development by increasing the morbidity and mortality of poultry. Colibacillosis is the most common bacterial disease of poultry. It is distributed worldwide and which is the major cause of morbidity and mortality with great economic losses in poultry industry. This disease caused by *Escherichia coli* which is very devastating gram negative organism. For the treatment of diseases, usually antibiotics are used in therapeutic dose. An antibiotic is a substance which is used in the treatment of disease either by killing the causative organism or prevents the growth of this organism. Beside therapy, indiscriminate use of antibiotics as metaphylaxis, prophylaxis, growth promoting in which sub-therapeutic doses of antibiotics leads to resistance of microbes against antibiotics (Schwarz et al., 2001). In Bangladesh, we have many medicinal plants which might be used for different diseases to avoid resistance of organism. The study was performed to show the antibacterial activity of henna

against *E.coli* for the use of extract of henna as a substitute of antibiotic. Based on the above introduction and discussion regarding on importance of medicinal plant I interested to do research on selective plants to study the antimicrobial resistance pattern of *E.coli* against commercial antimicrobial and to know the efficacy of *Lawsonia inermis* (henna) leave extract as a potential antimicrobials against *E.coli*.

CHAPTER: II
MATERIALS AND METHODS

2.1 Preparation of extracts:

Medicinal plants henna was collected from medicinal plant garden of Chittagong Veterinary and Animal Sciences University (CVASU) then washed, air dried and finally stored in Pharmacology laboratory, CVASU. Then the air dried samples were blended to yield fine powder after chopping. Fifty gram of each sample was taken and mixed with 500 ml of 95% methanol for 5 days at room temperature under dark condition. The suspension was shaken once in a day for 4 days in an orbital shaker (GFL®) and after 5 days, filtered the slurry through sieve. The suspension was filtered through Whatmann's filter paper No.1 (Whitman International Ltd, Maidstone, UK) then the filtrate was transferred to volumetric flask of rotary evaporator at 60 ℃ and 160 rpm up to formation of dry extracts. Finally the extracts were collected from volumetric flask by spoon and stored at 4 ℃ until further use.

2.2 Isolation of Bacteria:

A total of fifty (n=50) commercial broiler chicken were purchased from 4 different retail markets in Chittagong City. After sacrifice of these chickens, an opportunistic sample of liver from each bird was collected for isolation of *E. coli*. Bacteria was isolated after growing them in selective media as Mac Conkey agar (large pink color colony) and bacteria was preserved in fridge(-18℃) in by keeping at nutrient broth until further use. Five samples of *E. coli* taken and grown in blood agar.

2.3 Culture and Sensitivity Test:

Preparation of media

Mueller Hinton agar was used as culture media for antibacterial assay. Thirty eight gm of powdered media was dissolved in 1000 ml of distilled water in a conical flask and heated on flame to dissolve. Then the media was sterilized by autoclaving at 121° C temperature for 15 minutes.

Antimicrobial discs:

Six mm discs were made and kept them in a Pyrex bottle for sterilization in autoclave. 100 mg/µL, 75 mg/µL, 50 mg/µL and 25mg/µL of extract concentration was made by diluting the extracts with 2% DMSO (dimethylsulfoxide). Then each disc was soaked within 10 µL of diluted extracts.

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Standardization of bacterial concentration:

The concentration of bacteria was standardized with $BaSO_4$ turbidity standard which is equivalent to 0.5 McFarland Standard concentrations.

Bioassay:

By following Standard Disc Diffusion Assay adapted from Taylor *et al.* (1995) antimicrobial activity was determined. For this purpose, 20 ml of sterilized liquid media was poured in Petri dishes. By streaking method, bacterial culture was seeded in agar plate. Then the prepared antibiotic discs were placed on the media. Then the plates were incubated in the incubator by placing upside down at 37°C for 24 hours. Finally the inhibitory zone was measured by determining the diameter of the zone around the disc by measuring scale and then recorded accordingly.

2.4 Standard antibiotic discs for screening antimicrobial efficacy:

The standard antibiotic discs containing Ciprofloxacin, Enrofloxacin, Colistin sulfate, Tetracycline and Trimethoprim were used for measuring their antimicrobial efficacy against the tested microorganism. For the purpose of interpreting the antimicrobial sensitivity by measuring the diameter of zone of inhibition, following standard was followed:

Table 1: Standard measurement of diameter of zone of inhibition

SL	**Name of antimicrobial agent**	**Diameter of zone of inhibition(millimeter)**		
		Resistant	**Intermediate**	**Sensitive**
1	Ciprofloxacin	≤15	16-20	≥21
2	Enrofloxacin	≤14	15-17	≥18
3	Colistin sulfate	≤11	12-16	≥17
4	Tetracycline	≤11	12-14	≥15
5	Trimethoprim	≤10	11-15	≥16

Source: **CLSI, 2007; Seol *et al*., 2005; LO-Ten-Foe *et al*., 2007**

2.5 Statistical analysis:

Data were entered into the MS-Excel-2010. A descriptive statistical analysis was performed and the results were expressed as percentage, table and graph.

CHAPTER: III
RESULTS

The methanolic extract of medicinal plant L*awsonia inermis (*henna/mehedi)leave at different concentration (100mg/µl , 75mg/µl , 50mg/µl and 25mg/µl) were used to see the antibacterial activity against *Escherichia coli* which is isolated from colibacillosis affected chicken after postmortem.

Table 2: Sensitivity pattern of plant extracts against *Escherichia coli*

Type of bacteria	Name of sample	Zone of inhibition(in mm)			
		100mg/µl	75mg/µl	50mg/µl	25 mg/µl
Escherichia coli	1	Sensitive (18 mm)	Resistant	Resistant	Resistant
	2	Resistant (17 mm)	Resistant	Resistant	Resistant
	3	Sensitive (15 mm)	Resistant	Resistant	Resistant
	4	Sensitive (16 mm)	Resistant	Resistant	Resistant
	5	Sensitive (11mm)	Resistant	Resistant	Resistant

All sample of *E.coli* at 100mg/µl show sensitivity where as other concentrations show resistance to the entire samples. The highest zone of inhibition of extract is shown in sample number 1(18mm) and lowest zone of inhibition in sample number 5 (11 mm) at 100mg/µl concentration.

Table **3**: Zone of inhibition (mm) against *E. coli* for sample number 1

Extract	Control (ciprofloxacin)	Direct (5 µL)	Soaked	Disk (5µL)
Lawsonia inermis	25 mm	16 mm	18 mm	11 mm

Again extract of *Lawsonia inermis* was used in three different ways to test the level of sensitivity against *Escherichia coli* .The largest zone of inhibition (18 mm) was found with the disk soaked directly in the extract.

Table 4: The sensitivity and resistance pattern of different commercial antibiotics against *Escherichia coli*

Name of antibiotic	Zone of inhibition with sensitivity (mm)					
	Sample of *Escherichia coli*					S/I/R (%)
	1	2	3	4	5	
Ciprofloxacin	26 **S**	25 **S**	24 S	24 S	25 S	100%S
Enrofloxacin	19 S	21 S	20 S	19 S	19 S	100%S
Colistin sulfate	15 I	16 I	15 I	17 S	16 I	20%S 80%I
Tetracycline	09 R	11 R	10 R	11 R	11 R	100% R
Trimethoprim	12 I	10 R	10 R	11 I	12 I	40% R, 60% I

Where, 'S' – Sensitive, 'I' – Intermediary sensitive 'R'- Resistant

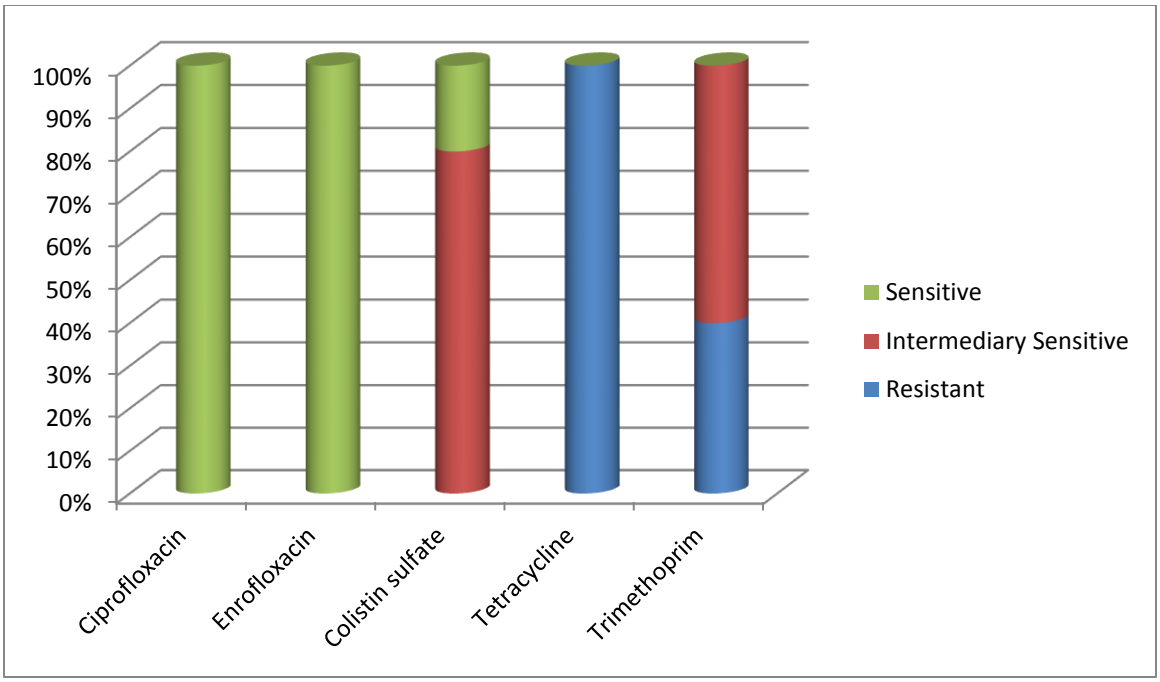
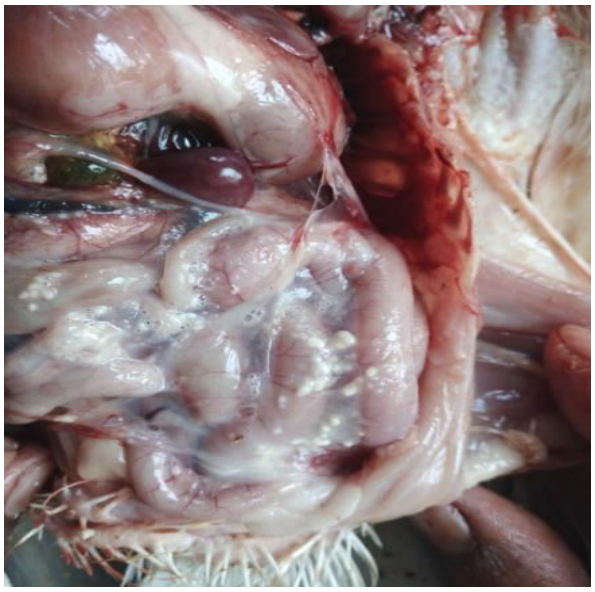
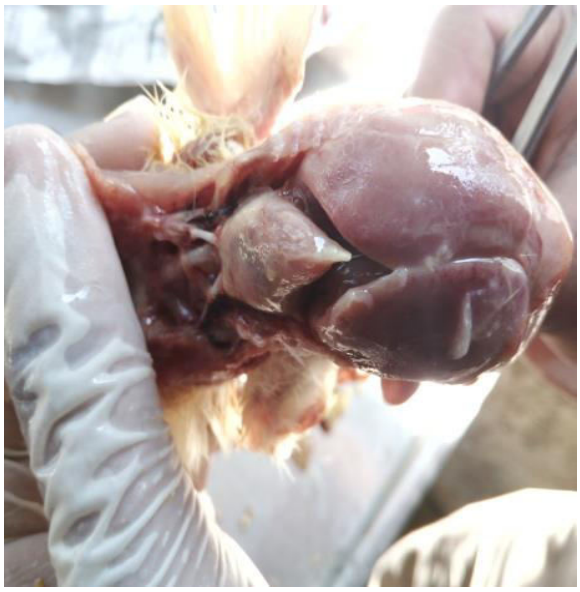


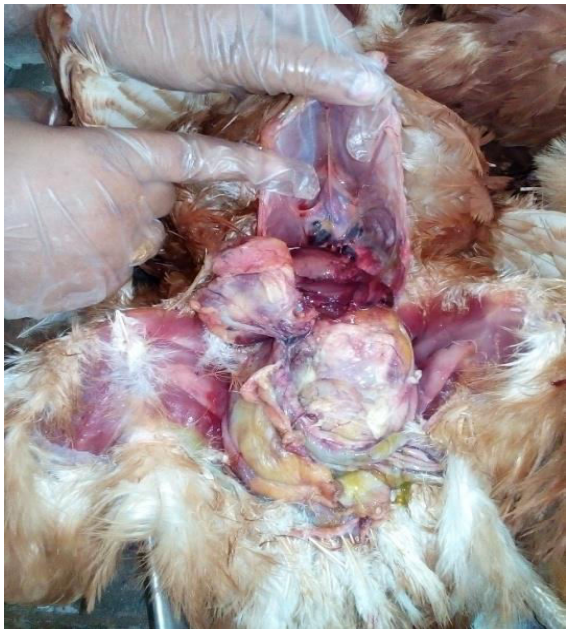
Figure 1: Graphical representation of the antimicrobial resistance pattern of the *Escherichia coli*



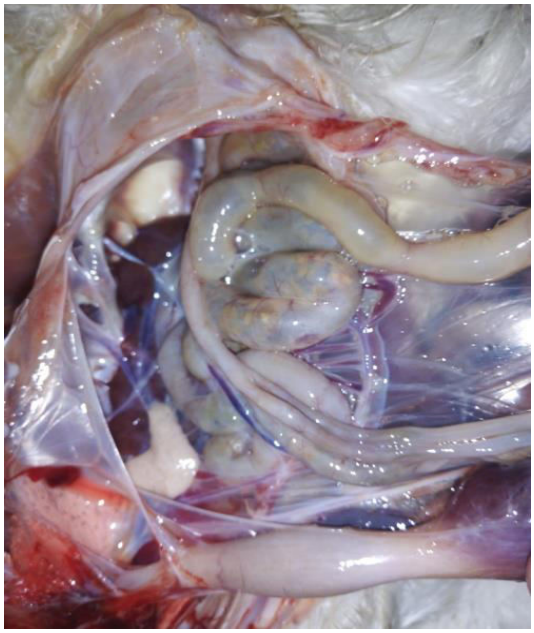
Airsacculitis form of colibacillosis



Perihepatitis form



Pericarditis form

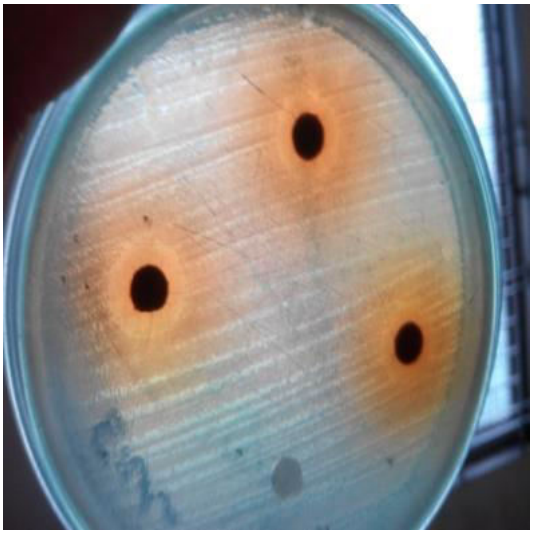


Airsacculitis form

Filtration of extract

Evaporation of methanol

Streaking of bacteria



Zone of inhibition against *Escherichia coli*

Figure 2: Some pictures of pathological and experimental work in laboratory

CHAPTER: IV

DISCUSSION

Antimicrobial agents are undoubtedly one of the most important therapeutic discoveries of the 20th century. However, with the 'antibiotic era' barely five decades old, mankind is now faced with the global problem of developing resistance in virtually all pathogens (Peterson and Dalhoff, 2004). Nowadays, not only human but also animal mostly poultry show the resistance against some popular antimicrobial. In recent years, many possible sources of natural antibiotics have been in use to cure several infectious diseases, mostly bacterial and fungal. In view of this, the searches for new antimicrobial agents from medicinal plants are even more urgent in the countries like India where in various infectious diseases commonly used antibiotics (Abebe *et al.,* 2003). Considering the high costs of the synthetic drugs and their various side effects to the health, the search for alternative drugs from plants used in folklore medicine is further justified. It is believed that plant chemical classes such as sterols, alkaloids, glycosides, saponins, flavonoids, tannins, and carbohydrates are generally superior in their antimicrobial functions (Cowan, 1999).

In my conducted study on the *E. coli* of commercial broiler chicken I have found Ciprofloxacin and Enrofloxacin as 100% sensitive against to those bacteria. We have found *E. coli* is showing 100% resistant against Tetracycline where as Trimethoprim is found to be 40 % resistant to test organism. No isolate has shown resistancy against Colistin Sulphate except some isolates have shown to be intermediary sensitive. In this study, Tetracycline was found to be 100% resistant against *E. coli*. Present study was agreed with the previous study, where the poultry *E. coli* isolates were found resistant to tetracycline (Biswas *et al*., 2001; Momtaz *et al.,* 2012; Hassan *et al.,* 2014). Trimethoprim showed 40% resistance against *E. coli* which is coincided with Reza et al., 2014 (39.62%) but lower than Bebora *et al*(1994) where they found it as 100% resistance and higher than Momtaz *et al.,* 2012 (29.8%).

This study has revealed Enrofloxacin as 100% sensitive against to *E. coli* but this is not similar as Gregova *et al.,* (2012) where they have found 43% resistance in *E. coli.* This may be due to not using Enrofloxacin as medication in that broiler farm. Ciprofloxacin showed 100% sensitivity against *E. coli* organism which is coincided with the previous study Rahman *et al.*, (2004). Nguyen *et al.*, (2016) indicated Colistin sulfate to be resistant against *E. coli* (24.4%) but

in my study I have not found any resistance rather 20% cases were sensitive and remaining 80% showed intermediary sensitive.

In my study, the highest zone of inhibition (18mm) at 100mg/µl concentration where as in antibacterial screening of *Lwasonia inermis* in human they found zone of inhibition which is nearest to the antibiotic they used, at 4000mg/µL concentration at well diffusion method (M. Nagarajan *et al.,* 2013). In antimicrobial screening *L. inermis* the highest zone of inhibition was 18 mm at 100 mg/µl concentration and all other extracts found resistant against *E. coli* whereas, the zone of inhibition was recorded 16 mm in methanolic extract of *Terminalia arjuna* in West Bengal (Dey *et al*., 2010) and 15.6 mm in methanolic extract and 15mm in ethanolic extract of *Terminalia arjuna* in Kurukshetra, India (Aneja *et al*., 2012) which are closely similar with my result. Methanolic extract of *Vitex negundo* (leaf and bark) show higher zone of inhibition (12.3mm) against *E.*coli at 25mg/dL (Thatoi and Dutta, 2009). In human the alcoholic and oily extract of *Lawsonia inermis* showed effective result against some pathogenic bacteria and fungus than the watery extract of henna. But in my study I only used methanol as the solvent because hexane, chloroform extract were not possible to perform in laboratory due to lack of sophisticated instrument. They also calculate the MIC(minimum inhibitory concentration) value of extract on various bacteria like *Strptococcus,* coagulase negative *staphylococcus, Pseudomonas aeroginosa*. (kathem k. *et al.,* 2008). I could not calculate the MIC of extract on *E coli* due to time constraint.

CHAPTER: V

LIMITATIONS

- The study period was short that's why it was not possible to do the experiment with large number of sample size.
- The active ingredients of plant were not studied due to constraint of time.
- Only methanol as solvent was used in the study.
- Could not able to trial all available antibiotic against *Escherichia coli* due to fund limitations.
- Could not be able to measure the level of antibiotic used as preventive in feed. Due to time limitations.

CHAPTER: VI

CONCLUSION

Escherichia coli isolate of broiler chicken is found resistant to tetracycline and only few are intermediary sensitive to trimethoprim .The methanolic extract of *Lawsonia inermis* (henna) leaf are tested sensitive to this bacteria in laboratory. The extract at 100mg/µl showed highest zone of inhibition (18mm) to this organism. So the extract of henna can effectively be used as the remedial measure of antimicrobial resistance to E.coli in broiler chicken. Further study will be needed to measure the efficacy of extract in *in vivo* condition.

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The author,
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BIOGRAPHY

Mosammat Moonkiratul Zannat is the daughter of late Delwar Hossain and Hosne Ara Begum. She passed Secondary School Certificate (SSC) in 2008 with G.P.A. 5.00 and Higher Secondary Certificate (HSC) in 2010 with G.P.A. 5.00. She admitted to Chittagong Veterinary and Animal Sciences University (CVASU) in 2011-2011 sessions. Now, she is an intern doctor of CVASU. As a veterinarian, she wants to focus on animal welfare and serve the nation. She has immense interest in research and conservation of wildlife.