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Surveillance of poultry farms and management of enterobacteria associated diseases through ethnoveterinary medicine

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SURVEILLANCE OF POULTRY FARMS AND MANAGEMENT OF ENTEROBACTERIA ASSOCIATED DISEASES THROUGH ETHNOVETERINARY MEDICINE

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in the Department of Botany, University of Rajshahi, Bangladesh

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DECLARATION

I do hereby declare that this PhD thesis entitled "Surveillance of poultry farms and management of enterobacteria associated diseases through ethnoveterinary medicine" has been originally carried out by me, under the guidance and supervision of Professors Dr. Most. Ferdousi Begum, Department of Botany, University of Rajshahi. This is the original work of mine and I declare that, I have never submitted the thesis or any part of this thesis for any other degree elsewhere.

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CERTIFICATE

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ABSTRACT

Twenty farms in Rajshahi Metropolis, Bangladesh were surveyed from January 2013 to December 2013 to assess the sanitation and hygiene quality of the poultry farms. The study results revealed the maximum disease incidence in January which was directly correlated with temperature and relative humidity and July was found the safer month for poultry production. In the waste management system, 85% of poultry farms scored 'not satisfactory' at a satisfactory level. Where a large number of coliform was recorded in different poultry feed samples. Total seven genera as *Escherichia coli* sp., *Klebsiella* sp., *Salmonella* sp., *Shigella* sp., *Enterobacter* sp., *Citrobacter* sp. and Proteus sp were identified from the poultry feed. Among the selected isolates only twelve isolates showed positive β-hemolytic activity. In the toxicity test through direct ingestion into the chicken, all the isolates showed positive results while in the Rose Bengal Agglutination test (RBAT) test only five bacteria showed strong agglutination reactivity.To *in vitro* control of pathogenic bacteria methanol, ethanol and acquous extract of six plants namely *Azadiracta indica*, *Carica. papaya*, *Zingiber officinale*, *Mentha. arvensis*, *Cynodon. dactylon*, *Peltophorum pterocarpum* were used . *C. papaya* showed the best performance for creating a zone of inhibition against *Shigella* sp. Considering the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) value, ethanolic extract of *A. indica* showed the best performance against the pathogenic bacteria. In the farm trial, it was remarkably noticed that *A. indica* leaf extract and *C. papaya* fruit extracts completely control the pathogenic bacteria and improved the live weight gain of the poultry about 11.16% and 11.37% higher than control, respectively. Thus the results suggest that strong policy measures are needed to improve the sanitary quality and change the attitude of poultry farmers and increase awareness of the causes and effects of potential outbreaks of poultry disease. Moreover, the continuous treatment with *A. indica* leaves and *C. papaya* fruit extract produced a significant increase in the live body weight and a significant effect on antibacterial activity. So, the effect of the selected plant extracts to be an important source in ethnovaterenirary medicine (EVM) practices to control entero-bacterial diseases of poultry and can be used as growth promoters that can minimize the unjudiciary uses of antibiotics in the poultry sector.

Dedicated to my beloved
Parents and Teacher

"In the name of Allah the most Merciful and Beneficent"

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(The Author)

CONTENTS **PAGE NO.**

CHAPTER ONE: INTRODUCTION 1-7

1.1 Poultry production and distribution in Bangladesh 2
1.2 Poultry feed used in Bangladesh 3
1.3 Major challenges in poultry production 3
1.4 Regulatory tools took in Bangladesh 3
1.5 The international regulatory authorities 4
1.6 Pathogenic bacteria in poultry 5
1.7 Controlling of pathogenic bacteria in poultry 5
1.8 Ethnoveterinary medicine (EVM) 6
Aims and objectives of study 7

CHAPTER TWO: REVIEW OF LITERATURE 8-20

CHAPTER THREE: MATERIALS AND METHODS 21-49

3.1 Survey 21
3.2 Microbial quality assessment of poultry feeds 26
3.3 Isolation of enterobacteria by using selected media 29
3.3.1 Characterization and identification of the isolates 30
3.3.2 Morphological or cultural features of the isolates 31
3.3.3 Biochemical test of the selected isolates 32
3.3.4 Identification of the selected isolated bacteria by Micro-Rao Online Software 38
3.4 Detection of pathogenic enterobacteria 39
3.4.1 Hemolytic activity test on tryptose blood agar 39
3.4.2 Virulence test of selected bacteria on poultry through direct ingestion 40
3.4.3 Rose Bengal Agglutination test (RBAT) of selected enterobacteria 40
3.5 In vitro Screening of plant extracts against selected pathogenic bacteria for antibacterial properties 41
3.5.1 Yield measurement of plant extracts 43
3.5.2 Phytochemical analysis of the selected plants 43
3.5.3 Analysis of phytocompounds by High-Performance Liquid Chromatography (HPLC) 44
3.5.4 In vitro antibacterial assay of selected plants 45

CONTENTS **PAGE NO.**

3.5.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)....47
3.6 Effect of neem leaf and papaya fruit extracts to control enterobacteria associated poultry diseases *(In vivo)*....47

CHAPTER FOUR: RESULTS....50-135
4.1 Survey....50
Recommendation....67
4.2 Microbial quality assessment of poultry feeds....68
4.3 Characterizations and identification of the pathogenic enterobacteria....72
4.3.1 Isolation of enterobacteria from poultry feeds....72
4.3.2 Biochemical test results of the selected isolates....75
4.3.3 Conformation of identification of the isolates with Micro-Rao online software....82
4.3.4 Confirmation of selected pathogenic bacteria....89
4.4 Pathogenicity test results of the isolated enterobacteria....90
4.4.1 Hemolytic activity test results on blood agar....90
4.4.2 Virulence test results of the isolates in poultry....92
4.4.3 Rose Bengal Agglutination Test (RBAT) results of the isolates....92
4.5 In vitro screening of plant extracts against selected pathogenic bacteria for antibacterial properties....95
4.5.1 Yield performance of the extract....95
4.5.2 Qualitative phytochemical analysis....97
4.5.3 Analysis of plant metabolites by HPLC (High-Performance Liquid Chromatography)....97
4.5.4 Evaluation of the antibacterial activity of plant extracts against the pathogenic enterobacteria....106
4.5.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of six plant extracts against selected pathogenic bacteria....123
4.6 *In vivo* trail of plant extracts against the pathogenic enterobacteria of poultry....129

CHAPTER FIVE: DISCUSSION....136-153

CHAPTER SIX: SUMMARY....154-157

CHAPTER SEVEN: REFERENCES....158-172

LIST OF TABLE **PAGE NO.**

Table 3.1 List of the surveyed poultry farms....23

Table 3.2 Types of mash foods for different types of poultry....24

Table 3.3 Amount of various components (in 100kg feed) which were mixed in different types of poultry feeds....24

Table 3.4 Coding of the isolates....30

Table 3.5 List of used standard phytocompounds and their Retention time (RT) with Reference....45

Table 4.1 Disease incidence of poultry in different poultry farms in and around Rajshahi city from January to December, 2013. 51

Table 4.2 Hygiene and sanitation status and room condition of surveyed farms.....61

Table 4.3 Hygiene practices, sanitary practices and satisfactory level of the poultry farms and workers 63

Table 4.4 Relationship of disease incidence with flocks, water sources and hygiene practices with 65

Table 4.5 Ranges of cfu/gm of poultry feed samples on different media 70

Table 4.6 Cultural characteristics of the isolates on MacConkey agar plate....72

Table 4.7 Cultural characteristics of the isolates on EMB agar plate....72

Table 4.8 Cultural characteristics of the isolates on SS agar plate....73

Table 4.9 Microscopic features of the isolates 73

Table 4.10 Biochemical test results of the selected isolates 79

Table 4.11 Hemolytic test results of the isolated enterobacteria in blood agar medium.... 90

Table 4.12 Virulence test results of the selected isolates in poultry 92

Table 4.13 Rose Bengal plate Agglutination Test (RBAT) result for the selected bacteria....93

Table 4.14 Yield of six plant extracts 95

Table 4.15 Qualitative test results of plant extract....97

Table 4.16 HPLC analysis result of *A. indica* leaves extract....99

Table 4.17 HPLC analysis result of *C. papaya* fruit extract....101

Table 4.18 HPLC analysis result of *Z. officinale* rhizome extract....102

Table 4.19 HPLC analysis result of *M. arvensis* leaf extract....103

Table 4.20 HPLC analysis result of *P. pterocarpum* bark extract....104

LIST OF TABLE **PAGE NO.**

Table 4.21 HPLC analysis result of *C. dactylon* leaf extract 105
Table 4.22 List of phytocompounds found (among used 12 standards) in studied plant extracts at a glance 106
Table 4.23 Antibacterial activity of *A. indica* leaf extract against the pathogenic bacteria of poultry feed using disc diffusion method..... 108
Table 4.24 Antibacterial activity of papaya (*C. papaya*) fruit extracts against pathogenic bacteria of poultry feed using disc diffusion method. 110
Table 4.25 Antibacterial activity of ginger (*Z. officinale*) rhizome extracts against pathogenic bacteria of poultry feed using disc diffusion method 112
Table 4.26 Antibacterial activities of mint (*M. arvensis*) leaves extracts against enteropathogenic bacteria of poultry feed using disc diffusion method . 114
Table 4.27 Antibacterial activities of Durba (*C. dactylone*) leaves extracts against enteropathogenic bacteria of poultry feed using disc diffusion method . 116
Table 4.28 Antibacterial activity of Radhachura (*P. pterocarpum*) bark extracts against entero-pathogenic bacteria of poultry feed using disc diffusion method..... 118
Table 4.29 Minimum inhibitory concentration (MIC) of six plant extracts against selected pathogenic bacteria..... 125
Table 4.30 Minimum bactericidal concentration (MBC) of six plant extracts against selected pathogenic bacteria..... 126
Table 4.31 Average live weight and weight gain (g) of Sonali chick in different treatments using *A. dirachta* leave extract. 130
Table 4.32 Average live weight and weight gain (g) of Sonali chicks in different treatments using papaya fruit extract 132

LIST OF FIGURE **PAGE NO.**

Fig 4.1 Comparison of percentage of gastrointestinal diseases (GID) in the surveyed area from January to December 2013 52

Fig 4.2 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 54

Fig 4.3 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 54

Fig 4.4 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 55

Fig 4.5 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 55

Fig 4.6 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 56

Fig 4.7 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 56

Fig 4.8 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 57

Fig 4.9 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 57

Fig 4.10 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 58

Fig 4.11 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 58

Fig 4.12 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 59

Fig 4.13 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%) 59

Fig 4.14 Relationship of disease incidence (DI) with types of flock 66

Fig 4.15 Relationship of disease incidence (%) in poultry flocks with water sources. 66

Fig 4.16 Relationship of disease incidence (%) in poultry flocks with personal hygiene status of the poultry workers. 66

	LIST OF FIGURE	PAGE NO.

Fig 4.17 HPLC peak profile of *A. indica* leaf extract 99

Fig 4.18 HPLC peak profile of *C. papaya* fruit extract 101

Fig 4.19 HPLC peak profile of *Z. officinale* rhizome extract 102

Fig 4.20 HPLC peak profile of *M. arvensis leaf* extract 103

Fig 4.21 HPLC peak profile of *P. pterocarpum* bark extract..... 104

Fig 4.22 HPLC peak profile of *C. dactylon* leaf rhizome extract 105

Fig 4.23 Comparison of zone of inhibition of *A. indica* leaf, *C. papaya* fruit & *Z. officinale* rhizome extracts (350mg/ml) against pathogenic bacteria. 121

Fig 4.24 Comparison of zone of inhibition of *C. dactylon*, *M. arvensis* and *P. pterocarpum* extracts (350mg/ml) against pathogenic bacteria..... 122

Fig 4.25 Comparative analyses MIC and MBC of six plants extract against the enteropathogenic bacteria 128

Fig 4.26 Comparison of MIC and MBC values against the enteropathogenic bacteria28..... 128

Fig 4.27 Comparison of three solvents against the enteropathogenic..... 128

LIST OF PLATE **PAGE NO.**

Plate 3.1 Location map of the studied areas 22

Plate 3.2 Photographs showing different stages of poultry in farm condition 25

Plate 4.1 Photographs showing gastro-intestinal disease symptom 52

Plate 4.2 Photographs showing liquid litter management system (A) & (B) and solid litter management system (C) & (D) in poultry farm. 64

Plate 4.3 Photographs showing poultry feeds and bacterial colonies on different medium: Poultry feeds (A-B); bacterial colonies on NA agar (C), MacConkey agar (D), EMB agar (E) and SS agar (F) plate. 71

Plate 4.4 Photographs showing different bacteria after Gram staining 74

Plate 4.5 Photograph showing Motility test (A), Catalase test (B), Citrate test (C), Indole test (D), Starch hydrolysis test (E.), Phenylalanine test (F) and MR test (G) 80

Plate 4.6 Photograph showing VP test (A), Oxydase test (B) and KIA test (C), Glucose fermentation test (D), Mannitol fermentation test (E), Sucrose fermentation test (F) and Lactose fermentation test (G) 81

Plate 4.7 Photographs showing pathogenicity test results: No hemolysis (A), β-hemolysis (B); direct ingestion of the hemolytic positive isolates in poultry (C), affected chicken (Dead) (D); RBAT test in micro-titer plate (E). 91

Plate 4.8 Photographs showing blood sample (immunized) (A); serum (B); antigen (C); RBAT test results in micro-titer plate (D). 94

Plate 4.9 Photographs showing different used plant parts and extracts. 96

Plate 4.10 Photographs showing antibacterial activities of *C. papaya* plant extracts. ethanolic (A), methanolic (B) and aqueous (C). 119

Plate 4.11 Photographs showing poultry feed, bacterial inoculum, plant extracts and additives used in *in vivo* condition. 133

Plate 4.12 Photographs showing experimental farms and measuring of treated chicken. 134

Plate 4.13 Photographs showing chicken under control and treatment 135

LIST OF SYMBOLS AND ABBREVIATIONS

The following symbols and abbreviations have been used throughout the text:

%	:	percentage
µ	:	micron
cm	:	centimeter
df	:	degrees of freedom
e.g.	:	exempli gratia, for example
et al.	:	et alli and others
etc.	:	etcetera, and so on
F	:	f- value from ANOVA probability table
Fig./s	:	figure/ figures
gm	:	gram
i.e.	:	id est=which to say in other words, that is
m	:	meter
mg	:	milligram
mg/l	:	milligram/liter
ml	:	milliliter
mm	:	millimeter
MS	:	mean square
no.	:	number
p^h	:	the negative logarithm of the hydrogen
sec	:	second
sp/spp	:	Species /species
SS	:	sum of square
v/v	:	volume by volume
viz	:	namely
P	:	probability of the data (or more extreme data) arising by chance,
SD	:	standard deviation
SE	:	the standard error(of sample mean) - a measure of uncertainty of statistic
SL	:	Serial number
^0C	:	degree centigrade

CHAPTER ONE
INTRODUCTION

Poultry is now a very important and widespread agricultural industry in the tropics (Sastry et al. 1983) which provides chief and high-quality protein to the nation that meets country demand. Now in Bangladesh, it has become a source of rapid profit, employment, and cheaper animal proteins. While China, Russia, and India's poultry industry growth ranked the world's top poultry producing countries, Bangladesh also has bright prospects in this field. Bangladesh's poultry sector has already targeted to export of egg and meat to the Middle East around 2024. With the increase in the number of the population, the demand for poultry meat and eggs will be double by 2021 from the current rate of poultry eggs and meat production. Thus the demand for meat, egg, and egg products have been expanding dramatically day by day with the increases in population and urbanization

To run successful poultry production in small scale commercial farms, sanitation and waste management practices should be deliberately practiced in day to day activities. Commercial poultry practices run at risk for poor sanitation systems. Proper efforts need to avoid disease outbreaks in poultry. To kill disease agents' proper sanitation equipment, housing, protective clothing for poultry workers should be needed. At the same time, several disinfectants and sanitizers should be used regularly to clean poultry houses. Moreover, following the entire program in prevention and control and strong monitoring take by regulatory authority it is not possible to keep a commercial poultry farm a hundred percent disease-free. Besides, contaminated water sources serving thousands of chickens in poultry farms can contribute in the occurrence of diseases that would certainly result in great economic losses.

Enterobacteriaceae is a large family of Gram-negative bacteria including many harmless symbionts and pathogens, such as *Salmonella*, *Escherichia coli*, *Klebsiella*, *Shigella*, *Enterobacter,* and *Citrobacter*. The gastrointestinal tract is the place of residence and transit of these microorganisms and carefully monitored by the immune system. When the feed is contaminated with these bacteria different types of gastrointestinal diseases can occur. The common gastrointestinal

diseases (GID) are *Bacillus cereus* infection, *Shigella* dysentery, Cholera, *Campylobacter jejuni* gastroenteritis, typhoid fever, Yersinia infection, Salmonellosis, *E. coli* infection, peptic ulcers etc. Many poultry is affected by a severe type of these infections and causes a death that creates great economic losses.

Ethnoveterinary medicine (EVM) is the use of plant material to care and treatment of cattle and poultry based on traditional knowledge. It is the common indigenous practice in many developed countries and the easy method of animal and poultry caring for successful poultry farming. It has already been established in Africa and other countries through trial and experiment. It can be valuable alternatives to allopathic medicine in animal husbandry and poultry farming. It can be great natural source of medication and poultry treatment in developing countries where conventional drugs are often not available to poultry farmers. It can play a vital role in the country's economic growth, where the government or non-governmental organizations aim to self-dependence and empower people by increasing the use of their knowledge and resources. Thus the EVM practices should be a meaningful means to control enter bacteria-induced diseases in poultry.

1.1 Poultry production and distribution in Bangladesh

The estimation of the poultry population in Bangladesh is depended on the source of information. According to numbers provided by the government of Bangladesh livestock department, about 195 million birds are produced in 2006. With the increase of the population, the demand of poultry is gradually increased day by day. The poultry farms are distributed all over Bangladesh but the commercial poultry farms are found largely in big cities of the country. Among the total production, fifty percent of poultry are produced in Dhaka and Rajshahi divisions. The aim of production systems was very different in these two divisions. In total poultry production, only 39.4% of commercial poultry are produced in the Rajshahi division while the Dhaka division it was 53.3% to fulfill the high requirements of the city’s people. In Bangladesh egg production also increases and

a total 5653 million eggs were produced in 2008. It is projected that there is a gradual increase in annual broiler meat production, which is almost triple since the last decade.

1.2 Poultry feed used in Bangladesh

In commercial poultry farming, different types of formulated feeds are used and the main ingredients are maize, rice bran, sesame oil cake, soybean oilcake, oyster shell, wheat husk, fish meal etc. The rates of mixing of these ingredients are varying in different feed companies to introduce into the market. All types of broiler feeds and only 18% of layer feed come from the feed meal industry and fifty percent of the raw materials in poultry feed are imported (Saleque, 2007).

1.3 Major challenges in poultry production

In Bangladesh, poultry farming is expanding up to rural areas which are independent and highly unorganized and mainly they used house yard for caring for poultry. In total poultry indigenous consist forty percent and the rest of the commercial farms. If the poultry farms in the countryside are well organized and improve their existing facilities farmers should be benefited. The productivity of commercial poultry is high compare to indigenous poultry but the survival rate is high in indigenous poultry due to genetic makeup. Commercial diets with additive and antibiotic supplements will increase productivity and reduce the chicken mortality rate making the company profitable (Sarker, 2013). Commercial poultry like broiler, Sonali, cock etc. are very much sensitive. For their caring well housing, special feed and intensive care are needed. Along with this additive and a large amount of antibiotics are used which increased production cost. Moreover, avian influenza and other enterobacterial diseases are commonly found which are greatly affected poultry production. For sustainable development of the poultry sector, the country should have a strong surveillance system (Haque et al. 2013) and experts' opinions should have taken by the government.

1.4 Regulatory tools took in Bangladesh

Poultry is Bangladesh's rising sector and has great scope for exporting the product to other countries. But there is still no established standard or organized

commercial poultry farm. In Dhaka and Chittagong city, only Aftab Poultry Ltd follows standard practices for poultry to marketing. Except these, no poultry farm follows international and national standards for poultry products marketing and practices. Bangladesh only has two laws such as the Animal Slaughter and Meat Act (1957) and the Municipal Corporation Ordinance (1983) to mitigate unsafe practices in poultry farming, there is no noticeable behavior witnessed by the regulators. Following the ratification of the Animal Disease Act and the Animal Products Quarantine Act in 2005, there is still no government initiative on feed health and poultry sanitation. Apart from these users, there is very low knowledge of food contamination and foodborne diseases. Like industrialized countries, consumer organizations cannot create pressure on poultry farmers to follow food-safety rules in Bangladesh. By the proper application of the existing policies and increasing the movement of regulatory authorities, this sector can be improved. It is difficult to establish policies without awareness of food safety and food safety threats and sources of financial support. Only by increasing awareness, overcoming financial constraints and improving the perception of consumers good quality of poultry products can be ensured to the consumed.

1.5 The international regulatory authorities

In developed countries feed markets have to follow some international rules. The FAO / WHO Codex Alimentarius Commission with its Hazard Analysis and Critical Control Point (HACCP) and the Codex Alimentarius Commission are the major international regulatory bodies developing international trade regulations or guidance. They also set guidelines for good agricultural practices (GAPs), good production practices (GMPs), and good hygiene practices (GHPs). These criteria were largely followed by developed countries for marketing in their own country and international markets. Also, the International Atomic Energy Agency (IAEA) also sets such standards for food and feed safety irradiation. Also, some international bodies such as the European Food Safety Authority (EFSA) and the African International Organization for Standardization (ARSO) also oversee and control some private industry.

1.6 Pathogenic bacteria in poultry

Salmonella, *Campylobacter*, *S. aureus*, *E. coli* and *Listeria* are potential pathogenic bacteria found in poultry and poultry meat. *Yersinia enterocolitica*, *Aeromonas* and *Clostridium perfringens* also have the potential to be significant pathogens in poultry, as well as common bowel and genital tract members of domestic animals, causing infection from raw poultry consumption (Stanley et al. 2003*).Campylobacter jejuni, C. coli* and *C. lari* are the most common bacteria in broiler meat (EFSA, 2010). *Campylobacter* is also isolated from poultry meat in Tamil Nadu and Calcutta (Varma et al. 2000). Many vectors like air, water, poultry workers, rodents, poor sanitation, etc are directly involved in cross-contamination of the feed that increases the number and types of the pathogen in the feed. By reducing the sources of contamination, poultry feed can be protected from unwanted pathogens.

1.7 Controlling of pathogenic bacteria in poultry

For the management of infectious diseases antibiotics are heavily used in the commercial poultry industry. There are different types of disease occurring through the manipulation of raw poultry or the consumption of contaminated poultry. Different antibiotics are tested against pathogenic bacteria, such as tetracycline, ampicillin, streptomycin, kanamycin, gentamicin, erythromycin, ciprofloxacin, etc. Although in *Campylobacter upsaliensis* no antibiotic resistance was known, most of the poultry isolates of *Campylobacter, Salmonell* and *Escherichia coli* were antibiotic-resistant. So, to solve these problems plant material can be effective means. Many researchers used plant materials to cure infectious diseases in poultry. Plant extracts, which are also used as growth promoters but also have antimicrobial, anti-inflammatory, antioxidant and antiparasitic activity (Hashemi et al. 2010). It is commonly considered safe and effective drugs to control pathogenic bacteria and is vastly used in traditional medicine. It is extensively used in feed to improve poultry production. In recent years, Asian, African, and South American countries extensively used plant extracts to control poultry diseases. Food pant additive supplementations have a

major influence on poultry growth and production efficiency (Hashemi et al. 2010).

1.8 Ethnoveterinary medicine (EVM)

Ethnoveterinary medicine (EVM) is a traditional animal health care culture in which natural plant materials are used for disease treatment (McCorke 1986). It has great importance in developing countries where poor farmers cannot afford the costly allopathic veterinary medicines. In a recent report, it is observed that farmers use traditional remedies for low cost in Nigeria (Chah et al.2009). *Aloe vera* is the most popular herb and is commonly used in Botswana and other countries for the remedy of poultry. Many poultry farmers in rural area use different medicinal plants,

In Africa and other developing countries, high incidences of disease hinder the development of poultry. For many days, the poor villagers are used EVM widely to control various poultry diseases based on their traditional knowledge. It is reported that 35% of villagers regularly used EVM in Nigeria, 58% used EVM in Tanzania, 59% in the Gambia, 79% in Botswana (Moreki 1997). Compared to other countries, only about 1% of Zimbabwe's poultry farmers use EVM (Kelly et al. 1994), although many plants have been used for care in animals since the 1970s (Chavunduka, 1976). The poultry farmers in the countryside are used EVM for prevention and curative measures of the diseases. In the Tabora and Morogoro regions of Tanzania, about 58% of village poultry farmers claim to use EVM to control poultry disease (Yongolo, 1996). Nevertheless, to use these remedial methods clinically, very few experiments have been carried out under controlled conditions. For example, the use of 10 g of *Kularzhoecrenate* leaves per liter of water has resulted in good results in domestic fowl prevention of avian coccidiosis (Agbédé et al. 1993). Therefore, in the present investigation, the efficacy of plant extracts against the pathogens was examined for successful ethnoveterinary practices.

Aims and objectives of the study

In recent years there has been a drastic change in the poultry sectors. Besides, with the growing awareness of the nutritional value of poultry products and their affordability, demand for poultry meat is rising, so the rearing of poultry has shifted to an industrial level. Farmers were encouraged to pursue poultry farming because of the strong adaptability characteristics of poultry. The United Nations Food and Agriculture Organization estimated that nearly 16 billion chickens were in the world in 2002 (FAO Study, 2006). Some adverse environmental factors have been identified for intensive poultry treatment and sometimes poor health conditions of the farmers can be the sources of disease and environmental pollution (Report from MoA, 2003). Considering the above point, the present study has been undertaken with the following objectives:

1. To survey the prevalence of enterobacteria associated poultry diseases of different farms in Rajshahi Metropolis and its surrounding areas.
2. To provide appropriate guidelines about hygiene and sanitation for healthy poultry farming.
3. To assess the microbial quality of the poultry feed to test the safety level of the poultry feed
4. To isolate and identify the enterobacterial isolates from the feeds.
5. To detect pathogenic enterobacteria through different serological tests.
6. To screening of plant extracts for antibacterial properties (*in vivo*).
7. *In vivo* trial of plant extracts to control pathogenic enterobacteria for development of ethnoveterinary medicine (EVM).

CHAPTER TWO
REVIEW OF LITERATURE

Survey

Poultry is an increasing sector in Bangladesh's agricultural economy, performing multidimensional functions such as nutrition, food supply, savings, power generation, income and manure, and daily expenses. But the unhygienic practices of farming, lack of proper sanitation system, microbial contamination of feed causes different types of diseases in poultry and economic losses. So, in this context, a brief review of literature pertinent to the present investigation was made from the work carried out by some of other researches country and abroad are given below:

Abbas et al. (2015) determined the prevalence of various poultry diseases in broilers and mentioned that the highest disease incidence in broiler as (7.85%) was observed in Newcastle disease (ND), in case of fowl typhoid (6.58%), Mycoplasma (5.68%), *Escherichia coli* infection (5.52%), Coccidiosis (4.59%), Mycotoxicosis (4.56%), Infectious Bursal Disease (2.84%), and Infectious bronchitis (1.59%) and also observed that the April to June was the safest month for broiler production.

Omojasola et al. (2015) analyzed commercial poultry diets in Nigeria and reported that the total bacterial counts in the chick mash ranged between 1.40 and 6.60×10^5 CFU g-1 and for the grower mash were 2.80 and 7.70×10^5 CFU g-1 and identified genera were as *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Klebsiella* sp., *Proteus* sp., *Pseudomonas aeruginosa*, *Escherichia coli*.

Talukdar et al. (2017) deected several diseases in Sonali as Bursal disease (IBD), Newcastle disease (ND), Coccidiosis, Colibacillosis, and Mycoplasmosis in 14.72% 11.24%, 13.95%, 14.72%, and 12.79%. On the other hand, commixed infection of IBD, ND, and colibacillosis was recorded in 15.89% of cases.

Khatun et al. (2018) surveyed poultry farms of seven divisions of Bangladesh and reported that 9.38% of farms followed the standard practice of bio-security, 41.48% of farms followed good practices, 32.53% of farms fell in the fair category and 16.59% of farms remained in the poor category.

Microbial quality assessment

Poultry offers nutritious food in the form of egg meat and job opportunities at different levels of human nutrition (Das, 1994). Poultry farming needs less investment compared to other livestock, small space becomes a more profitable and popular sector. So, there is no doubt, if the proper steps are taken by the government for brooding, breeding, disease control measures, and marketing in-country and abroad, it would be possible to develop the poultry industry to a great extent. For these reasons, microbial safety in poultry feed is the subjected of many investigations. Some of the relevant researches that have conducted on this context both in the country and abroad are given below:

De-Shalom (1999) reported that *Staphylococcus aureus* is the most predominant bacterial organism with 52 cfu (33%) followed by *Salmonella typhi* with 48 cfu (30%), the next bacterial organism isolated was *Bacillus cereus* with 40 cfu (25%) and the least was *Pseudomonas aeruginosa* with 18 cfu (12%) while the highest number of *S. typhi* was obtained also from the vital feed as 57 cfu per ml, followed by Guinea feed with 50 cfu per ml.

Ahmed (2010) analyzed contaminating poultry aliments in Khartoum State and recuperated 39.8% of isolates from victuals manufactured inside poultry farms, 33.2% from market victual, and 27.0% from inside aliment factories. According to indite of aliment, 50.2% of isolates were recuperated from broilers aliment, 32.2% from layers victual, and 17.5% from chicks victual. The isolates were identified as *Escherichia coli, Klebsiella pneumonia, Proteus vulgaris, Hafnia alvei, S. gallinurumin, K. oxytocain* and *S. pullorumin*.

Ezekiel et al. (2011) identified 5 genera as *Escherichia*, *Salmonella*, *Klebsiella*, *Enterobacter*, *Yersinia*; from the feed samples. *Escherichia coli* were the most dominating enterobacteria and the frequency was 80%. The rank of distribution of contaminating enterobacteria across feed types was chick mash > broiler starter > broiler finisher > growers mash > layers mash.

Okogum et al. (2016) analyzed poultry feed and determine the prevalence of bacteria as *Candida albcans*66.4%, followed by *Proteus* sp.18.6%, *Escherichia Coli* 10.6%, and *Pseudomonas* sp. 4.4%. The lowest microbial growths were observed in grower marsh 14.2%, layer and broiler finish marsh 15.9% each, chick marsh 25.7%, and broiler starter marsh 28.3% in ascending order.

Sule and Ilori (2017) analyzed poultry feed and reported that the ranges of cfu for bacteria were 5.0×10^3 to 1.76×10^6cfu/g while. The bacterial were identified as *Streptococcus salivarius, Streptococcus pyogenes, Micrococcus luteus, Micrococcus varians, Micrococcus roseus, Staphylococcus aureus, Staphylococcus saprophyticus* and *Staphylococcus hominis.*

Antimicrobial activity of plant extracts

Cheap and abundant sources of pharmacologically active substances are medicinal plants and are considered to be harmful to bacteria (Basile et al. 1999). Most synthetic drugs and growth promoters are combined with poultry diets for rapid growth in intensive farming, but their overuse causes adverse side effects on bird health and cancer to humans (Butaye et al. 2003). Scientists are now concentrating on the use of natural herbs and plants that can safely be used to improve the production of poultry. In this context, some reviews in the country and abroad are given below:

Tiwari et al. (2011) suggested that an aqueous solvent is useful for extraction of anthocyanins, starch, tannins, saponins, polypeptides, and lectins. Ethanol is better for tannins, polyphenols, polyacetylenes, flavonol, terpenoids, sterols, and alkaloids. On the other hand, methanol is good for anthocyanins, terpenoids, saponins, tannins, xanthoxyllines, totarol, lactones, quassinoids, flavones, phenones and polyphenols while acetone is better for phenols and flavonols.

Mostafa et al. (2017) reported that ethanolic extracts of *Punica granatum*, *Syzygium aromaticum*, *Zingiber officinales* and *Thymus vulgaris* were potentially effective against *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonellatyphi* while *Cuminum cyminum* was only effective against *S.aureus*. Ethanolic extracts of *P.granatum* and *S. aromaticum* were the most effective plant extracts and showed bacteriostatic and bactericidal activities against *S.aureus* and *P.aeruginosa* with MIC's ranged from 2.5 to 5.0 mg/ml and MBC of 5.0 and 10 mg/ml.

Recent years have seen a steady increase in the use of conventional medicinal plan ts for health care worldwide. Scientists are looking for new phytochemicals that co uld be used for the treatment of infectious diseases as a possible anti-

microbial agent. Today, almost 30% or more of modern pharmacological drugs are derived directly or indirectly from plants and are used in Ayurvedic medicines. Several researchers have tested plant antibacterial and antimicrobial properties to use them as alternatives to synthetic drugs for the treatment of infectious diseases (Cheruiyot et al. 2009). Increasing the bacteria's antibiotic resistance often requires new antibacterial drugs and natural targeted sources are conventional plants. Thus plants would be a specific natural source of antimicrobial drugs and provide fresh or lead compounds that may be used in the future global control of some

infections. Therefore, the present investigation has been conducted to screen phytochemical and antimicrobial properties of six medicinal plants namely a *Carica papaya*, *A. indica, Z. officinae*, *C. dactylon*, *M. arvensis* and *P. pterocarpum* and some reviews regarding these are given below:

***Carica papaya* L.**

Osato et al. (1993) screened antimicrobial activities of unripe papaya against *Escherichia coli*, *Salmonella* sp. and *Klebsiella* sp and determined the zone of inhibition 13.5, 8.0 and 10.5mm against *E coli*, *Salmonella* sp. and *Klebsiella* sp., respectively and MIC as 500mg/ml.

Doughari et al. (2007) investigated the antibacterial activity of *C. papaya* against *E. coli* and *Salmonella* sp. using the cup plate agar diffusion method. The zone of inhibition was determined as 8 and 14 mm against *E. coli* and *Salmonella* sp, respectively, and the MIC and MBC of the extracts were ranged between 50-200 mg/ml and 150 and 200 mg/ml, respectively.

Aguoru et al. (2015) evaluated the phytochemical and antimicrobial activities of three selected medicinal plants against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Salmonella typhi*, *Shigella flexneri*. The methanolic extracts inhibited the growth of tested microbes and the MIC value was determined as 12.5mg/ml against both *Salmonella* sp. and *Shigella* sp. and 25mg/ml against *E. coli* while MBC were 200mg/ml, and 100mg/ml for *Salmonella*.

Cimanga et al. (2015) screened antimicrobial activities of ripe and unripe fruits of *C. papaya* against *Escherichia coli*, *Klebsiella pneumonia*, *Klebsiellaoxytoca*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Shigella dysenteriae* and *Shigella*

flexneri and showed higher activity with MIC and MBC ranged from 7.81 to 250 µg/ml and 15.62 to 500 µg/ml, respectively.

Islam et al. (2015) evaluated antibacterial activities of the latex of *C. papaya* against *Escherichia coli and* exhibited strong antimicrobial activity having a zone of inhibition of average 14.66, 15.66, and 16 mm at the dose of 10, 15, and 20 mg/disk respectively.

Tumpa et al. (2015) searched antimicrobial activities of *C. papaya* against *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella typhi* and showed 5.0, 6.0 and 6.5 mm zone of inhibition at 15, 20 and 25 µl concentration, respectively against *Escherichia coli*. and 6.0, 7.0 and 8.5 mm zone of inhibition at 15, 20 and 25 µl concentration, respectively against *Salmonella* sp.

***Azadirachta indica* A.**

Biu et al. (2009) analyzed fresh matured neem leaves to screen for some active chemical constituents. Saponins had high scores in the extract, tannins and glycosides showed moderate scores, while alkaloids, terpenes, flavonoids, reducing sugars, pentoses and whole carbohydrates viewed low scores. Anthraquinones, ketones and monosaccharides were not detected from the extract.

Timothy et al. (2011) studied the active components and the antibacterial effects of the ethanolic leaf extract of *Azadirachta indica*. The result obtained from this study showed the presence of tannins, saponins, flavonoids, alkaloids, glycosides, reducing sugars and terpenes in the ethanolic leaf extract. The antibacterial effect produced by the extract was dose-dependent at the tested doses (6.25 mg, 12.5 mg, 25mg, 50 mg and 100 mg) on *Escherichia coli, Staphylococcus aureus* and *Salmonella typhi)*. The minimum inhibitory concentrations (MIC) for *E.coli*, S.

aureus and S. typhi were 2.39mg, 3.31 mg and 4.79 mg respectively. The effect produced by the ethanolic leaf extract at 100mg (18mm) was statistically significantly higher than that of 1.5 mg ciprofloxacin (14 mm) on *S. typhi* ($p<0.05$).

Lekshmi et al. (2012) measured neem extract's antimicrobial activity against *Streptococcus mutans, Streptococcus salivary and Fusobacterium nucleatum*. Neem organic extracts were prepared using various solvents including petroleum ether, chloroform, ethanol, and distilled water and screened for their antimicrobial activity. Among the four neem extracts, there was good antimicrobial activity against *S mutans* in petroleum ether and chloroform extract, 18 mm inhibition region at concentrations of 500 µg. Neem chloroform extract showed strong activity in the 18 mm inhibition zone against *Streptococcussalivarius*. *Fusobacterium nucleatum*, the third strain, was highly sensitive to both ethanol and neem water extract with 16 mm inhibition region.

Garima Pandey et al. (2014) studied the total phenol, flavonoid, and tannin content of *Azadirecta indica* and estimated as 1.03%, 5.33%, and 1.83% respectively. HPTLC studies revealed the presence of β-sitosterol, lupeol, rutin, ellagic acid, ferulic acid and quercetin in 50% ethanolic extract. The extract showed paramount antibacterial activity against *E. coli* and *S. aureus,* though it inhibited the magnification of *S. aureus* more efficaciously as compared to *E. coli.*

Francine et al. 2015) studied the antimicrobial profile of leaf and bark extracts of *Azadirachta indica*. Ethanol and aqueous extract of leaves and barks of *A. indica* (Neem) were tested against *Escherichia coli* and *Staphylococcus aureus*. Fresh neem materials were found to be the most exhibiting much effect on both

Escherichia coli and *Staphylococcus aureus* compare to neem leaves and neem barks.

Rasool (2017) evaluated the antimicrobial activity of *Azadirachta indica* and *Psidium guajava* at ethanolic extracts showed that all the combinations of extracts were effective against the test microorganisms (*Staphylococcus aureu, Salmonella typhi, E. coli* and *Pseudomonas aeruginosa*).

***Zingiber officinale* L.**

Ghasemzadeh et al. (2010) investigated the relationship between phenolics and flavonoids synthesis and photosynthesis efficiency within two Malaysian ginger (*Zingiber officinale*). High-performance liquid chromatography (HPLC) has been used for the detection and quantification of polyphenolic materials HPLC analytical results indicated that quercetin, rutin, catechin, epicatechin, and naringenin synthesis and partitioning were high in plants grown below 310 μmol $m^{-2}s^{-1}$.

Victoria (2012) has examined the antibacterial ability of various plant extracts namely as, *Zingiber officinale* (Ginger), *Azardirachta indica* (Neem), *Trachyspermum ammi* (Omum) against two pathogenic microorganisms *(E. coli* and *B. subtilis*) from poultry fecal sample by using a well diffusion method. The aqueous, ethanolic and methanolic extracts of the above said plants were prepared and tested. The result revealed that the methanolic and ethanolic extracts are potent in inhibiting *E. coli* and *B. subtilis.* The aqueous extracts of all medicinal plants are showed no inhibitory activity. The present study reveals that the poultry faucal samples should be maintained properly to prevent environmental pollution and also to inhibit the spread of infection. Moreover, if there may be infections

caused due to the poultry faucal organisms they can be controlled effectively by using the plant extracts studied

***Cynodon dactylon* (L.) Pers**

Chaudhari et al. (2011) researched six different organic solvents such as n-butanol, petroleum ether, methanol, ethyl acetate and chloroform to extract bioactive compounds from *Cynodon dactylon* leaves to test the antibacterial compounds against infectious disease-causing bacterial pathogens such as *Bacillus subtilis, Streptococcus pyogens, Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Proteus mirabillis* and *Pseudomonas aeruginosa* by paper disc method.

Cynodon dactylon's butanol extract was more successful against most of the test sp ecies. Ethyl acetate, methanol, petroleum ether, and chloroform were pursued to in hibit the growth of the studied organism.

Jazani et al. (2011) investigated *Cynodon dactylon* antibacterial activity against *S. aureus, A. baumannii, P. aeruginosa, Klebsiella* and *E. coli.* All isolates were sensitive to different concentrations of *Cynodon dactylon* root hydroalcoholic extract, with *P.aeruginosa* isolates being the most sensitive bacterial isolates to *C.dactylon* root extracts however 69% of isolates were ciprofloxacin immune. Results show that this herbal drug may be a new source of antimicrobial agents.

Rahman (2014) researched Cynodon dactylon's antimicrobial activity from three separate extractions (hot and cold aqueous extraction, extraction of methanol) against some of the gram-positive bacteria (*Staphylococcus epidermidis, Bacillus cereus*) and gram-negative bacteria (*Escherichia coli, Pseudomonas aeroginosa, Salmonella typhi, Shigella dysenteriae*) using the method of disc diffusion. Measured the diameter of the inhibition, region surrounding the disk. The *C.*

dactylon aqueous extract had antimicrobial activity against all test species suggesting broad extract spectrum activity. No easy zone was formed by extracting methanol.

Pandey et al. (2016) studied antibacterial activity of *C. dactylon* against *Escherichia coli* using Kirby–Bauer. Antimicrobial activity showed due to presence of bioactive compounds .The zone of inhibition compare with ciprofloxacin with different conc. (0.25mg,0.5mg, 1mg) no zone of inhibition activity, 18.23 mm / ± 0.671, 23.10 / ± 0.743mm for ethanolic extraction conc (0.5mg, 1mg), for aqueous extraction conc. (0.5mg, 1mg) zone of inhibition 6.84 mm / ± 0.485, 8.64 mm / ± 0.269.

***Mentha arvensis* L.**

Bupesh (2007) studied the function of antibacterial activity in *Mentha piperita* L. leaf extracts. Pathogenic bacteria such as *Bacillus subtilis, Pseudomonas aureus, Pseudomonas aerogenosa, Serratia marcesens* and *Streptococcus aureus.* The aqueou and organic leaf extracts have been found to have good antibacterial activity against a variety of pathogenic bacteria as revealed by the method of well diffusion in vitro agar. Mentha piperita's ethyl acetate leaf extract showed a marked inhibition compared to chloroform, petroleum ether, and water, with leaf extracts more on *Bacillus subtilis, P. aerogenosa* than *S. aureus, P.aureus* and *S. marcesens.*

Zhang et al. (2015) Studied the antibacterial action of *Mentha arvensis* ethanol extract against *Acinetobacter baumannii*. Disc diffusion and micro-dilution assays were used to determine the extract's antibacterial activity by calculating the extract's inhibition region, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the test bacteria.

Peltophorum pterocarpum

Jagessar (2007) researched *Luffa operculata*, and *Peltophorum pterocarpum*'s antibacterial activities of *S. aureus* (gram+ve), *E. coli* (gram-ve) and *C. albicans* use disc spreading stokes, pouring plate, spreading well and streak plate process. Three extractions of hexane, dichloromethane, ethyl acetate and ethanol were obtained from these extracts. The solvents were used as regulation, while ampicillin and nystatin were used respectively as sources for bacterial and fungal species. The solvents had no effect on the microorganisms while microbial growth was inhibited by ampicillin and nystatin. At 0.18 mg/10mL of the medium plate, all three plants showed antimicrobial inhibitory activity. Operation with the ethanol extracts was also the most common and the hexane was the least or zero. This study suggests extracts of *L. operculata* and *P. pterocarpum*can may be used in the regulation as herbal medicines.

Ravikumar and Rathinam (2009) tested hexane/acetone extracts of *Peltophorum pterocarpum*, *Colvillea racemosa* and *Bauhinia purpurea* of the Caesalpiniaceae family were tested against eight selected clinical isolates such as *S. aureus, S. epidermidis, P. aeuriginosa, K. pneumoniae, B. subtilis, S. marcescens, E. coli* and *P. fluorescens*. for antibacterial activity. Among the three plants, hexane extract of *P.pterocarpum* extract showed maximum inhibition against organism *K. pneumoniae* of 225 µg/mL concentration, and a minimum zone of inhibition was observed in acetone extracts of *C. racemosa* and B. purpurea of 75 µg/ml concentration against *E. coli*, respectively.

Effect of neem leaves and papaya fruit extracts in controlling of poultry diseases (*in vivo*)

No extensive work on the effects of plant extracts on body weight and performance in poultry has been done in Bangladesh. This study was conceived as

a possible alternative to antibiotic feed additives to determine the effect of a polyherbal extract on live weight. According to the situation of our country, Our farmer's awareness is very small because most of them are not properly trained in the development of broilers, but the unemployed young generation comes to this sector for a quick return on investment and benefits Pharmaceutical companies take advantage of this, convincing farmers to use antibiotics as a growth promoter or life savings for chicken. As a consequence, every broiler is an antibiotic depot. The antibiotic residue reaches the human body when these broilers are eaten by humans and cause serious human health hazards. An organism's resistance to antibiotics is one of the most important hazards. There is some concern over the use of human antibiotics to encourage growth in food-borne animals (Phillips et al. 2004). Different types of synthetic chemicals are used as a growth promoter and anthelmintic for treatment in broiler development such as antibiotics. Antibiotic growth promoters (AGPs) are antibiotics that are actively used to enhance growth and feed conversion in feed at a low level. They improve the performance, health status, and quality and efficiency of the production of birds. Growth promoters will certainly be successful. Nevertheless, there is growing public health concern about the use of antibiotics to promote growth in food animal production as a result of this user's contribution to rising antibiotic resistance levels.

Sharifi (2013) evaluated the results, blood lipid and microflora population effects of dietary supplementation of four medicinal plants in the ileum. In a fully randomized trial with 6 procedures and 4 replicates each, three hundred and thirty-six day-old Ross broiler chicks were used. The diets were iso-caloric and iso-nitrogenic, with dried cumin, peppermint, yarrow and poley herbs containing 15, 3, 2 and 2 g / kg respectively. The dietary supplementation of flavomycin and peppermint improved the broiler chickens ' FI and BWG relative to the control ($P<0.01$). By comparison to other nutritional therapies, dietary flavomycin

significantly increased body weight gain (BWG) ($P<0.05$). So, peppermint enhanced growth efficiency under the conditions of this study and adding it to the diet could be an alternative to using antibiotics as growth promoters in poultry development.

Mostofa (2013) tested the effectiveness of neem (*Azadirachta indica*), nishinda (*Vitex negundo*) and papaya (*Carica papaya*) powdered drinking water supplement as a growth promoter in poultry. A total of 40 day-olds Cobb 500 broiler chicks were bought from the local hatchery (Nourish Poultry & Hatchery Ltd.) and randomly divided into two classes, A and B, after seven days of acclimatization. Group A has been developed as a control and not treated. Group B was supplemented with feed powder with feed and water with neem, nishyinda and papaya dried leaves. The initial body weight of groups A and B on the 7th day of the experiment was 130±3.56 gm and 145±4.35 gm respectively and 1450±47.35 gm and 1600±58.56 gm respectively after the 42nd day of the experiment; the total body weight gain was 1320±43.79 gm and 1455±54.25 gm respectively.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Survey

In the present study, different poultry farms in and around Rajshahi Metropolis were surveyed from January to December 2013. The survey was mainly conducted to ascertain the sanitation condition of the farms and hygiene consciousness of the workers and as well as their waste management system to reduce the contamination of the environment and to protect public health.

Methods

The survey was conducted based on some structural questionnaires. The following questionnaires were used when the farms were visited:

1. Details of poultry farms
2. Workers condition
3. Hygienic status of workers
4. Hygienic and sanitation conditions of the poultry farms
5. Types of sanitizers used
6. Sources of water
7. The environmental condition of the farms
8. Poultry diseases
9. Number of total poultry and affected poultry
10. Waste disposal system

Selection of study areas

Different places of Rajshahi Metropolis and surrounding areas were selected for the study. The main considerations in selecting the study areas were as follows:

(a) A large number of poultry farms were raised in these areas;

(b) This type of study was not conducted previously;

(c) Well-communication system of the farms;

(d) Satisfactory co-operation from the farm owners and

(e) Lacking personnel awareness of poultry workers.

Selection of poultry farms

A total of twenty large size commercial poultry farms were selected for the survey. The main consideration in selecting the poultry farms was as follows:

a) The selected poultry farms covered the area in Rajshahi Metropolis;
b) A large number of commercial poultry were nourished in these farms.
c) Well, behavior of the poultry farm owners.
d) The communication system of the poultry farms was well.

The poultry farm owners were interviewed personally and data were collected on the interview schedule. A list of surveyed poultry farms is shown in Table 3.1. Types of feed using different aged flocks (Table 3.2) and the number of various components of poultry feed (Table 3.3) were presented.

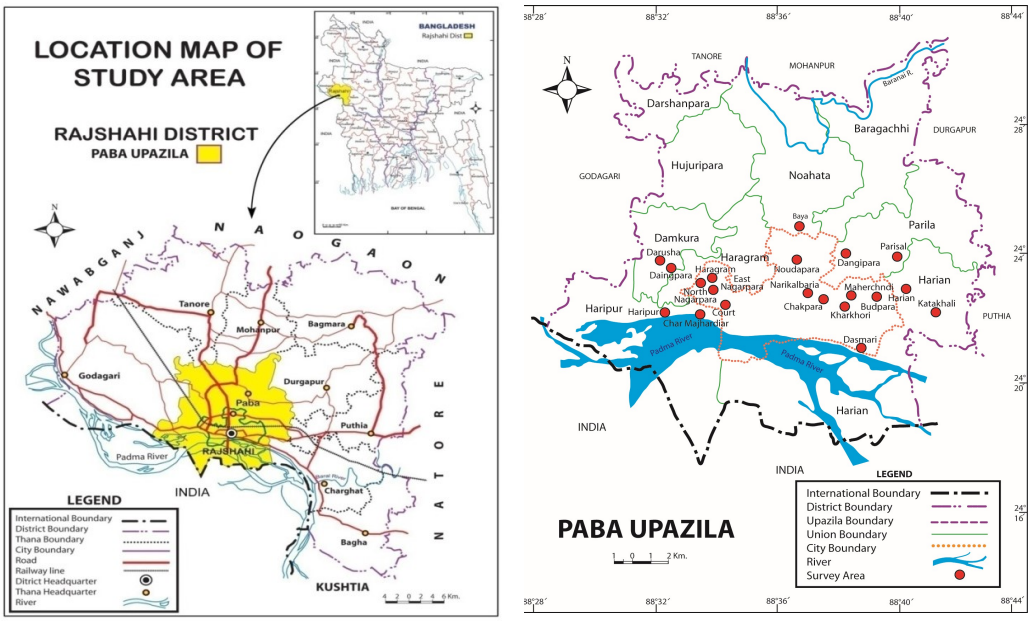

Plate 3.1 Location map of the studied areas

Table 3.1 List of the surveyed poultry farms

SL no.	Name of poultry farm	Feed used	Name of nourished poultry	Aim of nourished
1	Ismail, Coart	Stater/grower	Sonali	Meat
2	City, Nogarpara east	Stater/grower	Sonali	Meat
3	Amar, Nogarpara n	Stater/grower	Sonali	Meat
4	Rakib, Daingpara	Stater/grower	Sonali	Meat
5	Mahbub, Meherchondi	Stater/grower	Sonali	Meat
6	Peyarul, Khorkhory	L.Stater/ grower/ finsher	Layer	Egg and Meat
7	Juwel, Chokpara	Stater/grower	Sonali	Meat
8	Ahadur, Narikelbaria	Stater/grower	Cock	Meat
9	Belal, Noudapara	L.Stater/ grower/ finsher	Layer	Egg and Meat
10	Ahab, baya	Stater/grower	Cock	Meat
11	Iffat, Dangipara	Stater/grower	Sonali	Meat
12	Sakil, Parisal	L.Stater/ grower/ finsher	Layer	Egg and Meat
13	Sojib, Horipur	Stater/grower	Sonali	Meat
14	Bulbul, Haragram	Stater/grower	Sonali	Meat
15	Masum, Darusa	Stater/grower	Broilar	Meat
16	Khokon, Chormajardiar	Stater/grower	Cock	Meat
17	Rosid, Budpara	Stater/grower	Cock	Meat
18	Mostak, Dasmari	L. Stater/ grower/ finsher	Layer	Egg and Meat
19	Sohidul, Katakhali	Stater/grower	Sonali	Meat
20	Saon, Horian	Stater/grower	Sonali	Meat

Table 3.2 Types of mash foods for different types of poultry

Name of feed	Types of poultry	Age
Broiler starter ration	Broiler	0-4 weeks
Broiler finisher ration	Broiler	After 4 weeks to finish
Layer starter ration	Layer	0-8 weeks
Layer grower ration	Layer	9-20 weeks
Layer finisher ration	Layer	After 20 weeks to finish

Table 3.3 Amount of various components (in 100kg feed) which were mixed in different types of poultry feeds

Name of components	Name of poultry feed				
	Layer starter	**Layer grower**	**Layer finisher**	**Broiler starter**	**Broiler finisher**
Maise	50-52%	48-43%	43-45%	48-52%	46-50%
Rice bran (automatic mill)	14-23%	15-22%	18-21%	18-22%	19-23%
Sesmae oil-cake	4-10%	5-10%	5-10%	4-11%	6-11%
Soybean oil-cake	9-16%	9-11%	7-12%	8-16%	8-12%
Oyster shell	0-2%	2-3%	7-9%	1-3%	1-3%
Wheat husk	0-4%	5-10%	5-44%	0-3%	0-3%
Fish meal	4-11%	8-12%	6-13%	5.5-13%	6.5-13%
Cockcidio test *	0-10%	-	-	-	-
D.O.T*	-	50 g	500 g	50 g	50 g
Salt *	50 g	50 g	-	500 g	500 g
Primax –L*	50 g	500 g	300 g	25 g	-
Primax –G.S*	500 g	-	-	25 g	-
D.C.P*	300 g	-	250 g	500 g	500 g
Lysene *	500 g	250 g	100 g	50 g	500 g
Metheonine *	100 g	500 g	50 g	50 g	100 g

*Additional components were added to the above poultry feed

Plate 3.2 Photographs showing different stages of poultry in farm condition

Determination of temperature and relative humidity in the sampling site: Digital hygrometer was used for the recording of temperature and relative humidity of the selected poultry farms month wise.

Determination of disease incidence (%): To determine the disease incidence (DI) of poultry in different farms, each month the total number of poultry and affected poultry were counted month wise. Then the (DI) percentages were calculated by applying the following formula:

$$\text{Percentage of DI} = \frac{\text{Affected poultry}}{\text{Total poultry}} \times 100$$

Statistical analyses

Data and information on poultry farming and workers were collected and soon afterward were up-loaded on to a PC for further processing. Data were compiled, tabulated and analyzed following the objectives of the research. Microsoft Excel spreadsheets and SPSS for Windows (version 10.0) were used for analyzing the experimental data.

3.2 Microbial quality assessment of poultry feeds

For microbial quality assessment of poultry feeds, the samples were collected from different commercial poultry farms of Rajshahi Metropolis and surrounding areas from January to December 2013.

Sample collection

The poultry feed samples were collected in sterilized polybags and tied to the open portion of the bags by rubber band properly. Then the samples were immediately taken into the laboratory and held for further analysis at 4^0C.

Labeling of the samples

After collection, the samples were labeled according to their sources (Table 3.4).

Media used for detection of microbial quality of poultry feed samples

After the serial dilution technic according to (Reiner, 1982), different types of media were used for counting the bacterial load from poultry feed samples that are presented below:

Nutrient agar medium

Nutrient agar is used for the cultivation of bacteria and for the enumeration of bacteria of dairy products, various foods, and other materials. To prepare nutrient agar medium Downers and Ito (2001) method were followed with the following composition:

Components	Amount (gm/L)
Beef Extract	3.0
Peptone	5.0
Nacl	5.0
Agar	15.0

Preparation: Beef extract 3.0 g, peptone 5.0 g, NaCl 5 g, agar 1.0 g have been blended gently in 1L distilled water. Then heat with constant agitation and cook for 1 minute to dissolve the powder completely and autoclave it. The agar was cool to 45-50 ° C after autoclave mixture and pour into Petri dishes. Then it was solidifying for at least 30 minutes. When the medium becomes solid the Petri dish were prepared for culture and stored at below 4°C.

MacConkey agar medium

MacConkey agar is a versatile and differential culture medium for the isolation and differentiation of gram-negative bacteria and for the fermentation of lactose. The growth of gram-positive bacteria is inhibited by bile salts and crystal violet. Due to the pH shift, bile salts may also precipitate out of the media surrounding fermenter development. Non-fermenters may create colonies that are usually colored or gray.MacConkey agar was prepared using the following composition (Downes and Ito, 2001; Food and Drug Administration, 1995).

Components	Amount(gm/L)
Peptone	17.0
Protease peptone	15
Sodium chloride	5.0
Lactose	10.0
Bile salt No. 3	15
Neutral red	0.03
Crystal violet	1.0
Agar	13.5

Preparation: MacConkey agar was taken in a beaker with 1000 ml of distilled water at first 50.1 g and blended well. The prepared medium was disposed of by

separating the funnel into conical flasks (500 ml) and the prepared medium was autoclaved for 15 minutes at 121 ° C. It was cool to 50-55 ° C after autoclave liquefies agar and pour into Petri dishes. Then it was solidifying for at least 30 minutes. When the medium becomes solid the Petri dish was prepared for culture and stored at below 4°C.

Eosin Methylene Blue (EMB) medium

EMB agar is a solid medium that is very versatile. Originally developed for the differentiation of Escherichia coli and Aerobacter aerogenes by Levine, it proved effective for the rapid identification of Candida albicans and was found to be useful for the identification of coagulase-positive Staphylococci (Health Protection Agency, 2007).EMB medium prepared using the following composition:

Components	Amount (gm/L)
Peptone	10.0
Lactose	10.0
Dipotassiummonohydrogenphosphate	2.0
Methylene Blue	0.065
Eosine Y	0.4
Agar	15.0

Preparation: Peptone 10.0g, lactose 10.0g, di-potassium mono-hydrogen phosphate 2.0g, methylene Blue 0.065g, eosin Y 0.4g, and agar 15.0g was dissolved in 1L of distilled water and mix thoroughly. Then heat with frequent agitation and boiled for 1 minute to completely dissolve the powder and autoclaved at 121°C for 15 minutes. The liquefied agar was cool to 45-50 ° C after autoclave and pour into Petri dishes. After autoclave liquefy agar was cool to 45-50°C and pour into Petri dishes. Then it was solidifying for at least 30 minutes. When the medium becomes solid the Petri dish were prepared for culture and stored at below 4°C.

Salmonella-Shigella (SS) Agar medium

SS agar (Salmonella-Shigella Agar) is a differential selective media used for the isolation of *Salmonella* sp. and some *Shigella* sp. from pathological specimens, suspected foodstuffs, etc. SS medium prepared using the following composition:

Components	Amount (gm/L)
Beef extract	5.00
Peptic digest of animal tissue	5.00
Lactose	10.00
Bile salts mixture	8.50
Sodium citrate	10.00
Sodium thiosulphate	8.50
Ferric citrate	1.00
Brilliant green	0.00033
Neutral red	0.025
Agar	15.00

Preparation: First, 63.02gm SS agar has been suspended and boiled with regular agitation in 1000 ml distilled water to fully dissolve the medium. Overheating will kill medium selectivity, so it has not been done to overheat or autoclave. Then cool the average around 50°C gently and pour into the Petri plates.

Plating of the medium

Firstly, 20 ml of each medium was poured on a sterilized Petri plate and rotated smoothly to make a thin layer of medium. Then wait until the medium converts to solid. Initially, Nutrient ager, MacConkey, EMB, and SS agar were used for counting bacterial numbers and separation of bacteria from poultry feed samples. In Nutrient agar, the micropipette dropped EMB, SS and MacConkey agar diluted from each sample and the glass spreader expanded the sample. Para film sealed the plates and incubated them for 24 hours at 37°C. Bacteria growths have been detected through the colonies ' different characteristics.

Enumeration of cfu

After the incubation period, the Petridis contain various colonies chosen to be counted. Counting of colonies was normally done by visual observation. Then total cfu were determinate according to the following formula:

$$\text{cfu} = \frac{\text{Number of Colony}}{\text{Number of dilution (used to make plating for colony count)}} \times \text{Amount plated}$$

3.3 Isolation of enterobacteria by using selected media

For enterobacteria isolation, 1 ml of each suitable sample was exuded by the micropipette and spread by the glass spreader on the previously prepared EMB, SS, and MacConkey agar plate. The para-film taped the Petri dishes and incubated

them for 24 hours. Based on their morphological characteristics, separate colonies were chosen for isolation. The selected colonies were marked and their characteristics were analyzed according to different viewpoints such as the isolated strains were leveled according to these characteristics elevation, margin, and air, color, etc. (Eklund and Lankford, 1967). Then in Petridis, the marked and observed colonies were aseptically transferred to the fresh nutrient agar medium. From the plates, morphologically distinct colonies were collected and coded by source.

Labeling of the isolates

The isolated pure cultures were coded conforming to the sample's origin and the serial no of the samples used (Table 3.4). These code numbers were maintained for further use.

Table 3.4 Coding of the isolates

Farm name	Samples code	Isolates code
Sakil, Juwel,Mahbub, Ismail,Mostak, saon, Pearul, Juwel, Mahbub, Ismail, Sojib, Mostak, Rakib, Juwel, Ahab, Belal,Saon,	SGE, JSE, MLE, ISE, MSE, SSE, PLE, JGE, MJE, IGE, SGE, MGE, RGE, JSE, AGE, BSE, SGE	SGE-1, JSE-2, MLE-3, ISE-4, MSE-5, SSE-6, PLE-7, JGE-8, MJE-9, IGE-10, SGE-11, MGE-12, RGE-35, JSE-36, AGE-37, BSE-38, SGE-39
Rakib, Juwel,Ahab, Saon, Mahbub,Mostak, Pearul, Rakib, Ahadur, Juwel, Rakib, Mahbub	RGS, JSS, ASS, SGS, MGS, MLS, PLS, RGS, AGS, JGS, RSS, MSS	RGS-13, JSS-14, ASS-15, SGS-16, MGS-17, MLS-18, PLS-19, RGS-20, AGS-21, JGS-22, RSS-23, MSS-24
Rakib, Ahab, Pearul,Mahbub, Juwel, Mostak, Khokon, Ismail, Juwel, Rakib, Juwel, Mahbub, Pearul, Mostak, Ismail	RGM, AGM, PLM, MLM, JSM, MSM, KSM, IGM, JGM, RSM, JSM, MGM, PLM, MSM, ISM	RGM-25, AGM-26, PLM-27,MLM-28 JSM-29, MSM-30, KSM-31, IGM-32, JGM-33, RSM-34, JSM-40, MGM-41, PLM-42, MSM-43, ISM-44

3.3.1 Characterization and identification of the isolates

Characterization and identification of the microorganisms is a continuous process, which includes a series of different types of experiments. The following procedure was carried out for the probable identification and characterization of the selected isolates.

3.3.2 Morphological or cultural features of the isolates

The bacterial colonies grown on cultural pate containing different media (EMB, SS, and MacConkey agar) were studied as to their color, shape, edge, elevation, margin, etc. whether grown inside at the bottom or on the surface of the media in pour plate condition. The characteristics like turbidity, sedimentation, surface growth if any (flocculent, ring, pellicle and membranous etc.), and distribution of growth in the broth culture were observed and recorded.

Broth culture characteristics

The selected microorganisms were inoculated in a nutrient broth medium. The characteristics of growth into broth like turbidity, sedimentation, surface growth if any (flocculent, ring, pellicle and membranous etc.), and distribution of growth in the broth were observed and recorded.

Microscopic Observation

Observation of cell morphological characteristics by a microscope is an important step to identify an unknown bacterial strain. The structure of vegetative cells was microscopically studied and microphotographs were taken by a camera fitted with a microscope. All the Data were collected which was decided by microscope from different characteristics like rod, chain or cluster etc.

Gram Staining

For Gram staining, Koby and Ronald's (1985) modified method was followed. Gram staining was demonstrated using the following reagents:

Reagent
Crystal violet solution (1%)
Lugol's Iodine solution (1% iodine in 2% KI)
Ethanol (95%)
Safranine (1% for counter stain)

Procedure: A small drop of sterile water was mounted on a clean microscope plate. A part of a young colony was extracted from the agar medium with a cold and sterile loop. Through moving the slide four times through a Bunsen flame, the bacterium was air-dried and heat-fixed on the slide but was not overheated. The slide was crystal violet overflooded and set aside for 60 seconds. The slide was then rinsed under running water and drained off excess water. Then it was flooded with iodine solution and set aside for 60 seconds. After washing with 95% ethanol

for 30 seconds it was rinsed with water. The slide was then blotted with safranine for 10 seconds, dried, and counter-stained. Then it was dry and water rinse. At X40 magnification, the slide was eventually analyzed using a light microscope with oil immersion. The presence of crystal violet or dark purple color indicated the Gram-positive and Gram-negative bacteria were tested by safranine or red color.

3.3.3 Biochemical test of the selected isolates

To identify and classify isolated bacteria, the following biochemical test was performed.

Motility test

This test is used to determine if an organism is motile or non-motile, because, to identify an unknown bacterium it is usually necessary to determine whether the organism is motile or not. For the motility test, Health Protection Agency (2007) with a slight modifications method (Hanging Drop Method) was followed.

Typical composition of the motility test medium

Components	Amount (gm/L)
Tryptose	10.0
Sodium Chloride	5.0
Agar	5.0

Procedure: The motility test medium was demonstrated the swimming ability of bacteria in a semisolid medium. In a straight-line stab with a needle, a semi-solid medium such as 0.75% agar is inoculated with the bacteria. After incubation, if turbidity (cloudiness) can be observed away from the line of the stab due to bacterial growth, there was evidence that the bacteria could swim through the medium.

Catalase test

Catalase testing was conducted to confirm the microorganism's ability to release gas (O_2) from hydrogen peroxide (H_2O_2) through enzymatic (catalase) reaction. According to MacFaddin (1980), a catalase test was performed.

Procedure: For this purpose, a small amount of test organism was taken from agar plate or slant culture onto a slide. Added a few drops of hydrogen peroxide to the

smear. The production of bubbles confirms (these would be O_2 bubbling up) the test is positive.

Indole test

Indole test was taken according to Holt et al. (1994). This test demonstrates the ability of certain bacteria, accumulating in the medium, to decompose the amino acid tryptophan to indole. Indole is then tested with a p-dimethyl-amino benzaldehyde calorimetric reaction. For the indole test, tryptophan or peptone broth medium and Kovac's reagent were used.

Media preparation: In 1 liter of purified water, all ingredients were dissolved and thoroughly combined, and heated to dissolve. It was then dispensed and autoclaved at a pressure of 15lb / $inch^2$ at 121 ° C for 20 minutes. The media pH was adjusted to 7.0.P-dimethyl-amino benzaldehyde was dissolved in amyl alcohol and then added to the HCl acid slowly. This reagent was stored at 4^0C and was shaken gently before use.

Procedure: Tubes of tryptophan broth were inoculated with the test organism from an agar culture plate and incubated for 48-72 hours at 37^0C. Sometimes 96 hours was required for optimum accumulation of indol. In the broth culture, a few drops of Kovac's reagent have been added. The indication of a positive reaction was a red color in the alcohol layer.

Citrate test

Depending on the use of citrate, Simmons Citrate Agar is used to distinguish microorganisms. For the separation of enteric Gram-negative bacilli, Simmons Citrate Agar is recommended.

Principles: In Simmons Citrate Agar, ammonium dihydrogen phosphate is the only source of nitrogen. Phosphate Dipotassium acts as a buffer. Sodium chloride maintains the medium's osmotic balance. Bromthymol Blue is the pH indicator. Organisms that can utilize Ammonium Dihydrogen Phosphate and Sodium Citrate as their sole sources of nitrogen and carbon will grow on this medium and produce a color change from green (neutral) to blue (alkaline). Agar is the solidifying agent.

Procedure: For the citrate test, 3ml of the citrate medium of koser was brought into a test tube and infused at 37^0C for 4days with a fresh culture of test strain and incubate. Observed medium growth by turbidity and changed color, an indicator of light green to blue (alkaline reaction).

Phenylalanine determination test

To determine the capability of some organisms to take out the amino group ($-NH_2$) from amino acids.

Typical composition of phenylalanine agar (pH 7.3)

Components	Amount (gm/L)
Yeast extract	3.0
Dipotassium Phosphate	1.0
Sodium chloride	5.0
Bacto agar	12.0

Procedure: All the chemical compounds were diffused in 1 liter of distilled water and mixed gently. The mixture was heated to dissolve the compounds. Then the medium was carried out for autoclaved. Using sterile technique, inoculate each experiment organism into its appropriately labeled tube using a streak inoculation. Incubate cultures at 37ºC for 24 to 48 hours.

Methyl Red (MR) and Voges-Proskauer (VP) test

Using methyl red and Voges-Proskauer reactions, MR-VP broth was used to separate bacteria. This is a positive response from Voges-Proskauer. MR-VP Medium, MR-VP Broth (2008) used these test methods.

MR-VP broth medium

Typical Composition of MR-VP broth medium (pH 7.0± 2.)

Components	Amount (gm/L)
Buffered Peptone	7.0
Dipotassium Phosphate	5.0
Dextrose	5.0

Procedure: Within 1 liter of distilled water, all chemical compounds were diffused and mixed gently. To remove the chemicals, the mixture was heated. It was subsequently dispensed and autoclaved for 20 minutes at 121°C.

Methyl red indicator

Components	Amount (gm/ml)
Methyl red	0.1
95% ethyl alcohol	300
Purified water	500

Preparation: The MR indicator was prepared by dissolving 0.1 g of methyl red in 95% ethyl alcohol in 300 ml. Added enough filtered water to make 500 ml.

Voges-Proskauer test reagent

Reagent A

Components	Amount
Alpha napthol	5 g
absolute ethyl alcohol	95 ml

Reagent B

Components	Amount (gm/ml)
Potassium hydroxide	40
Creatine	0.3
Distilled water	100

Procedure

Methyl red test: 5 drops of methyl red indicator was added to an aliquot of the broth.

Voges-Proskauer test: In 1 ml of broth culture reagent A 15 drops and reagent B 5 drops were added by the dropper. To aerate the sample the mixture was mix well after the addition of each reagent.

Oxidase test

An Oxidase test was carried out for the enzyme oxidase of our unknown isolates. For the oxidase test, the Goszczynska et al. (2000) method was followed.

Typical composition of the oxidase test medium

Components	Amount
N,N,N'N-tetramethyl-ρ-Phenylenediaminedihydrochloried	1g
Distilled water	1000 ml

Media preparation: Media was prepared with 1 gm of N,N,N'N--ρ-phenylenediaminedihydro-chloride and dissolved in 100 ml of distilled water.

Procedure: For the oxidase test wet filter paper method was followed. For this test a few drops of N,N,N',N'– tetramethyl-ρ-phenylenediamine dihydrochloride solution was placed on a new piece of Whitman No, 1 filter paper with a clean Pasteur Pipette. Remove part of a colony with a sterile toothpick. Then smear was done onto the moistened paper and color was changed to dark purple within 30 seconds was positive and it was taking longer up to 60 seconds, for widely positive.

Kligler's iron agar (KIA) test

Kligler iron agar is well known for gram-negative bacteria, particularly Enterobacteriaceae. KIA reactions are based on lactose and glucose fermentation (dextrose) and hydrogen sulfide production.. Kegler's iron agar (KIA) test was carried out according to Kligler (1917) with slide modification.

Typical composition of KIA test medium

Components	Amount (gm/L)
Peptone	23
Yeast extracts	3
Glucose	1
Lactose	10
Iron (II) sulfate	0.2
Sodium chloride	0.1
Sodium	0.3
Thiosulphate Phenol red	0.05
Agar	7

Media preparation: 49.6 g of media composition were suspended in 1 liter purified water and heat until dissolved completely. Autoclaved at 121^0C for 15 minutes and cool down to 4-5^0C.Added urea solution 40% (TN1308) per liter of culture medium (final urea concentration: 2%). Shake well and put into decontaminated tubes. Allow setting as slant agar with approximately equal length of butt and slant.

Procedure: To inoculate the medium of KIA; a straight wire loop was used. First, the ass was stabbed and then the slope was streaked in a zigzag pattern and for 24

hours incubated at 350C. The tubes ' cotton plugs were kept loose. Therefore, the bacteria are both exposed to an anaerobic (butt) and aerobic (slant) environment. As an indicator, phenol red is present. Through aerobic degradation of protein components in the medium to alkaline materials, if the bacteria are non-fermenters, they will expand on the slant. The slant and the ass should stay red. When dextrose, but not lactose, can be fermented by the bacteria, acid is formed in the slant and the butt, and the yellow medium. Nevertheless, the dextrose will be used up within 12 hours. Through degrading proteins, bacteria continue to grow at the surface. From eighteen to twenty-four hours, the alkaline end products allow the medium to revert to red. If both lactose and dextrose can be fermented by the bacteria, after prolonged incubation, the slant and butt will remain yellow. Notwithstanding the production of alkaline products by protein degradation, the high concentration of lactose holds the slant acidic. As for indicators of the production of hydrogen sulfide, KIA also contains sodium thiosulfate and ferrous sulfate.

Starch hydrolysis test

Starch agar is a differential medium that measures an organism's ability to produce certain exoenzymes that hydrolyze starch, including a-amylase and oligo-1,6-glucosidase. Starch molecules are too large to reach the bacterial cell, so some bacteria secrete exoenzymes that can then be used by the organism to dissolve starch into subunits.

Typical composition of the starch hydrolysis test medium

Components	Amount (gm/L)
Beef extracts	3
Agar	20
Soluble starch	2

Procedure: Starch agar is a simple nutritive medium with starch added. Media composition was suspended in 1 liter distilled water and heat until completely dissolved. Autoclaved for 15 minutes at 121^0C and inoculum from a pure culture is streaked on a sterile plate of starch agar with 24 hours incubation of the inoculated plate at 35-37^0C. Iodine is then applied to the growth surge. Clear halos around colonies are positive for their ability to digest starch and therefore indicate

the presence of alpha-amylase. A clearing around the bacterial growth indicates that the organism has hydrolyzed starch.

Glucose Fermentation Test

The test is used to differentiate the organisms that ferment glucose and these are the members of Enterobacteriaceae. The test method was done according to Cheesbrough (2000)

A typical composition for Fermentation test

Compositions	Amount
Gelatine Peptone	10 g
Sodium Chloride	5 g

Sucrose Fermentation Test

The test is used to differentiate the organisms that ferment sucrose and the test method was done according to Cheesbrough (2000)

A typical composition for Fermentation test

Compositions	Amount
Gelatine Peptone	10 g
Sodium Chloride	5 g

Mannitol Fermentation Test

The test is used to differentiate the organisms that ferment mannitol and the test method was done according to Cheesbrough (2000)

A typical composition for the Fermentation test

Compositions	Amount
Gelatine Peptone	10 g
Sodium Chloride	5 g

3.3.4 Identification of the selected isolated bacteria by Micro-Rao Online Software

Some software is used for the identification of microorganisms which are alternative to commercial systems, code-books, or identification tables. Micro-Rao online Bacterial identification Software (http://www.microrao.com) is the most popular and effective software for the identification of enterobacteria. Based on morpho-biochemical characters, cultural characteristics, and growth conditions

etc. data the software gives probable bacteria names. This database contains an encyclopedia dedicated to plenty of bacterial species. For use of the software, at first, the webpage was opened where a chart will appear with sixteen biochemical test results. Then the chart options (positive, negative or unknown) were filled with the biochemical test result of desire isolate. Once the relevant information was given, the program showed the isolate's genus with the percentage of probability.

3.4 Detection of pathogenic enterobacteria

Pathogenicity is an organism's ability in another organism to produce infectious disease. Pathogens are disease-causing infectious agents, including bacteria, viruses, fungi, protozoa, and higher parasites. The term is used to describe the capacity of microbes to cause disease. For virulence test of the selected isolates hemolytic activity test, toxicity test on poultry, and RBAT were conducted.

3.4.1 Hemolytic activity test on tryptose blood agar

Tryptose blood agar base was used with blood for determining the hemolytic reactions of fastidious pathogenic microorganisms. Four forms of hemolysis on blood agar media such as Alpha (α), Beta (β), Gamma (γ), and Alpha-prime (α′). This test was carried out according to Harmon et al. (1995). Blood agar was prepared using the following composition.

Components	Amount (gm/L)
Tryptose	10.0
Beef Extract	3.0
Sodium Chloride	5.0
Agar	15.0

Medium preparation: 33 g of the components were suspended in 1L of purified water and mixed well. Then heated and boiled for 1 minute to completely dissolve the powder. After that, the medium was autoclaved at 121°C for 15 minutes. 5-10% sterile defibrinated blood aseptically added at 45-50°C and mixed well in medium to produce a blood agar medium. Then the medium poured into Petri dish.

Procedure: Loop full of fresh bacterial broth cultures were streaked on blood agar. The cultural plates were incubated aerobically at 37^{o} for 18-24 and 48-hour

incubation. Then the prepared culture blood agar medium was observed for growth and hemolytic reactions.

3.4.2 Virulence test of selected bacteria on poultry through direct ingestion

Virulence test of selected isolates was carried according to Rat Pyometra Model (Mikamo et al. 1998) with slide modification. For this test a constant dose of one ml (6.9×10^9cell/ml) for each isolate were ingested in poultry and sterile normal saline was ingested in control. Each group consisted of three poultry with 9 to 13 weeks old and weight was 460-658 gm. The experimental period was continued for 15 days and weight loss was measured after 3 days' interval. The sickness and mortality of the poultry were monitored for each treatment. Percentages (%) of weight losses after 15 days were determined for each poultry by the following formula:

$$\text{Percentage (\%) of weight loss} = \frac{\text{Final weight}(W_2) - \text{Initial weight}(W_1)}{\text{Initial weight}(W_1)} \times 100$$

3.4.3 Rose Bengal Agglutination test (RBAT) of selected enterobacteria

Pathogens, dead cells, or complex cell products when invaded into the body, it induces formed antibodies and these substances are called antigens. When blood is taken from an immunized animal serum fraction contains antibodies, such antibody-containing serum is termed antiserum or immune serum. The reactions (*in vitro)* between antigens and antisera form the basis of serological techniques.

Preparation of antiserum: Antisera are produced by oral ingestion of poultry with selected bacterial isolates in 3 days intervals up to 15 days. After immunization blood samples were collected with the needle from the wing vein. This blood sample was kept 5 minutes at normal temperature for clotting. Then the samples were centrifuged at 1000rpm for 5 minutes and the clear antiserum were separated and kept into a small vial.

Preparation of antigen: Antigen was prepared from the five selected isolates for the Rose Bengal Agglutination test (RBAT) following a modified method of Annonymus, (2004). At first, the selected strains were cultured in a culture tube with NA broth for overnight at 37^{o}C. Then the cultures were preserving by added 1ml of phenol saline (0.5% phenol in 0.85% sodium chloride solution) to each

tube and gently agitated. From this bacterial suspension equal volume (0.5ml) of culture and 1% Rose Bengal dye (1:1) were taken in an Eppendorf tube. The organism died by heating at 80ºC in a water bath for 1 hour. The concentration of bacteria cells was adjusted to 6.58×10^{9}/ml, Macfardland constant (OD). Then the suspension was centrifuged at 10000 rpm for 15 minutes. The sediment was resuspended in sterilize saline water (0.5% NaCl) and stored at 4ºC.

Test procedure

At first 0.5ml antiserum and an equal volume of saline taken into Eppendorf tube and serially diluted up to 4^{th} fold. Then the aliquot (30µl) of each fold was transferred into a micro-titer plate serially for each bacterial strain. After that equal volume (30µl) of Rose Bengal antigen were added and in the case of control only saline water was used instead of antiserum. The mixture was rocked gently for 4 minutes at ambient temperature and then observed for agglutination. Any visible reaction is considered to be positive and scored as the following scale (Annonymus, 2004):

++++ = Agglutination with a prominent / rose-colored flakes appearance with full (100%) mixture clarity

+++ = Ring / smaller flakes of incomplete (about 75%) mixture clarification

++ = Partial ring / small flakes with a mixture clarity of about (50%)

+ = Dark ring / small grain agglutination, negligible mixture clarity (approximately 25%).

- = No agglutination

In the presence of distinct agglutination with the appearance of prominent ring / small or large flakes with the reactivity of 50 to 100% (+ + to + + +), the reaction was considered positive and it was negative if graded at less than + +.

3.5 In vitro Screening of plant extracts against selected pathogenic bacteria for antibacterial properties

Plant Materials Used

In the present investigation, six plant extracts were used to determine the antibacterial activities against five selected pathogenic bacteria. The name of the plants and their used parts were as follows:

Botanical name	Family name	Parts used	English name	Bengali name
Azadirachta indica A.	Meliaceae	Leave	Neem tree	Neem
Carica papya L.	Caricaceae	Fruit	Papaya	Pepe
Zingiber officinale R.	Zingiberaceae	Rhizome	Ginger	Ada
Cynodon dactylon L.	Poaceae	Leaves	Grass	Durba
Mentha arvensis L.	Lamiaceae	Leaves	Mint	Pudina
Peltophorum pterocarpum L.	Fabaceae	Bark	Yellow gold mohor	Holudkrisnochura

Collection of Plant Materials

The plant materials used in this investigation were collected in different seasons for their availability. These materials were collected locally from Shaheb Bazaar and Rajshahi University area, Rajshahi, Bangladesh, and were identified and authenticated by Dr. AHM Mahbubur Rahman, Professor, Botany Department, Rajshahi University, Bangladesh.

Solvents used

For extraction of plants, three solvents (aqueous, ethanol and Methanol) were used as below:

Solvents used for extraction

Name of Solvents	Molecular formula	Boiling point	Melting point	Density
Water	H_2O	99.974°C (373.124 K) (211.95°F)	0°C (273.15K) (32°F)	0.998g/cm^3
Ethanol	C_2H_6O	78.4°C, 352K, 173°C,	-114.3°C, 159K, -174°C	0.7918 g/cm^3
Methanol	$CH_{30}H$	65°C	-97.6 °C	0.791 g/cm^3

For extraction Parekh and Chanda (2007) method was followed. Each of the plant samples powder was impregnated in aqueous and ethanol solvents separately for 7 days with continuous shaking in a shaker (Model No. KS 250 JANKE & KUNKEL). After 7 days dissolving materials were filtered with tettron cloth into a beaker and final filtration by filter paper. Then the filtrated extraction was evaporated using water bath. Finally, dried spices extracts were obtained in different amount each of plant samples and then stored at 4°C in air tight screw-cap tube.

Storage of plant materials

The freshly collected plant materials were washed with distilled water to remove dirt and then chopped into pieces and dried at room temperature (32-35^{o}C) continuous for five days until a constant weight was obtained. 250 g of each part of the plant is heavily powdered with a mortar and pestle and further reduced to powder with an electric blender. Then the powdery materials were stored at 4^0c for further use.

Formulation of extracts from plants

Plant extracts were prepared with slight modification using Sultana et al. (2009) methods. Each of the powdered air-dried plant material was extracted with methanol or ethanol or aqueous solvent separately. 5 g of each powdered sample was mixed with 100 ml of organic solvent (i.e. methanol, ethanol, and aqueous) in a conical flask and then allowed to soak for 24 hours at ambient temperature. The extracts were then filtered with tetron cloth into a beaker and then using Whatman no. 1 filter paper. The filtrates were concentrated at 40^0C using a rotary evaporator (EYELA, SB-651, Rikakikai Co. Ltd. Tokyo, Japan). In a screw cap tube, the condensed extracts were then collected and stored for further use at 4^0C.

3.5.1 Yield measurement of plant extracts

Yield of different plant extracts (semi solid) was calculated according to Sultana et al. (2009) following the formula:

$$\text{Yield (\%)} = \frac{\text{Yield (g)}}{\text{Used plant material (g)}} \times 100$$

3.5.2 Phytochemical analysis of the selected plants

Qualitative tests for phytochemicals were conducted according to Harborne (1998). The crude powder of the six plants samples i.e. *Azadirachta indica*, *Carica papya, Zingiber officinale, Cynodon dactylon, Mentha arvensis* and *Peltophorum pterocarpum* were used and followed procedures are given below-

- **Test for alkaloid**

  For the alkaloid test, 200 mg of crude powder for each plant sample emerged in 10 ml methanol separately. Then 2 ml of filtrate was taken for each sample and added 1% HCI. The stream was carried out then 1 ml filtrate and 6 drops of Mayor's reagents. A ceramic precipitate was observed.

- **Test for tannins**

  For the tannin test, 200 mg of crude powder of each plant sample emerged in 10 ml distilled water separately and 2 ml of filtrate was taken in a test tube. Then added 2 ml of $FeCl_3$. A blue-black precipitate was observed.

- **Test for saponin**

  For the saponin test, 200 mg of crude powder of each plant sample emerged in 10 ml distilled water separately and 0.5ml of the filtrate was taken of each plant and added 10 ml distilled water in a test tube. Then the test tube was vigorously shaken. Then frothing and the emulsion was observed.

- **Test for steroid**

  For the steroid test, 200 mg crude powder of three types of plant samples emerged in 10ml chloroform separately. Then 2 ml filtrate, 2 ml acetic acid, 50% concentrated H_2SO_4 were added in a test tube. A greenish color in the lower chloroform layer was observed.

- **Test for flavonoid**

  For the flavonoid test, 200 mg crude powder of each plant sample was emerged in 10 ml ethanol separately and filtrated and 2 ml filtrate was taken in a test tube and a few drops of ferric chloride (10%) were added. Then a green or blue color was observed to indicate the presence of the phenolic nucleus (Brain 1975).

3.5.3 Analysis of phytocompounds by High-Performance Liquid Chromatography (HPLC)

HPLC analysis was performed to detect and measure extract Phyto compounds using Gradient HPLC System (Waters Corporation, Milford, Massachusetts, USA) with Empower^{2TM} software system fitted with Waters 515 HPLC pump and Waters In-line degasser AF and D2 lamp photodiode detector. The retention time of each metabolite was detected with a Waters 2489 dual absorbance detector (Waters Corporation, Milford, Massachusetts, USA) at 280nm wavelength. Empower2™ software was used for integration and calibration. The evaluation was via peak areas with linear regression. The purity of the compounds was checked by comparisons between standards and samples with the area under the graph value.

For preparing plant sample, selected plant parts (freshly collected) were ground in mortar pestle using deionized water and were centrifuged at 1200G (G = Earth's

gravitational force) for 10 min before separating the supernatant in Eppendorf tubes and storing the supernatant at -20^{o}C for further use.

The standard stock solution was prepared by methanol to take a calibration curve. Sigma-Aldrich, Co., St. Louis, and Carl Roth obtained the formula for each product. A solution was prepared in methanol with a concentration of 10 µl for each plant extract. All solutions (both standards and samples) were filtered before injection or HPLC analysis using 0.22 µm Minisart Syringe Filters (Sartorius Stedim Biotech, Germany). In this investigation retention time, (RT) of some standard compounds (Oxalic acid, Ascorbic acid, Gallic acid, β-sitosterol, Glycine, Cysteine, Caffeic acid, Siringic acid, Phenylalanin, P-coumaric acid and Citrate) were calculated in our Laboratory following the Empower2™ software and for rest of the compounds, RT was followed by Zilani et al. (2016). The retention times (RT) of used phytocompounds (standard) were as follows:

Table 3.5 List of used standard phytocompounds and their Retention time (RT) with Reference.

Name of phytocompounds used as standard	Retention time (RT) of standard phytocompounds	Reference
Oxalic acid	2.7,3.3.7	Calculated
Ascorbic acid	4.56	Calculated
Gallic acid	6.10	Calculated
β-sitosterol	7.45	Calculated
Glycine	12.42, 13.2	Calculated
Cysteine	12.73	Calculated
Vanillic acid	20.09	Zilani et al. (2016)
Caffeic acid	20.83	Calculated
Siringic acid	21.18	Calculated
Vanillin	24.05	Zilani et al. (2016)
Phenylalanine	24.61	Calculated
Citrate	28.02,30.7	Calculated

3.5.4 In vitro antibacterial assay of selected plants

Antibacterial activity of the different plant extracts was evaluated against selected pathogenic bacteria using the disc diffusion method according to Bauer et al. (1966) with slight modifications.

Test organisms

Five selected pathogenic bacteria were used for this investigation namely *Escherichia coli*, *Klebsiella*sp, *Enterobacter*sp, *Salmonella* sp. and *Shigella* sp.

Preparation of different concentrations of plant extracts

The yield of different plant extracts was reconstructed in methanol, ethanol and aqueous for different concentrations at the rate of 150, 200, 250, 300, and 350mg/ml from the mother extractions (5g in 100ml) by the respective solvent.

Disc Preparation

Disk preparation was carried out followed by Bauer et al. (1966). The 6 mm size diameter disc was made with Whatman no. 1 filter paper using a punching instrument. Such disks have been cleaned within a screw-capped tube in an autoclave. Then the sterilized disc was absorbed with selected extracts by the parameter of 10 µl and taken at a normal temperature about 15 hours to dry the disk. Prepare disc was coded confirm to specific plant extracts and concentrations. After that, the coded disc paper was stored at 4^0C in a vial for further experiment.

Antimicrobial sensitivity test

The pathogenic bacteria were first inoculated into tubes of nutrient broth separately and incubated at 37 ^{0}C for 18 h. To standardize the inoculum density, McFarland constant 0.5 (1.5×10^8cell/ml)was used then glass spreader inoculated the nutrient agar plates uniformly with test species and rotated the plate 600 each time to ensure even inoculum distribution. The plate was then allowed for 3 to 5 minutes, but not more than 15 minutes. Then, earlier prepared paper disks were placed on the surface of the inoculated agar plate uniformly (no closer than 24 mm from center to center). On a plate of 90 mm, no more than 5/6 disks. A disk should not be shifted once it has come into contact with the agar surface as some of the compounds diffuses almost instantaneously. The plate was inverted and incubated at 37 ° C for 24 hours after positioning the disk. The plate was then examined and the diameters of the clear zones on the surface of the agar including the disk diameter were determined. The zones were a measure to the nearest millimeter using a ruler and values <8 mm were considered as not active against microorganisms according to Zhu et al. (2005), Koroch et al. (2007), Chang et al. (2008), Nayan and Shukla(2011) and Vijayarathna

et al. (2012). To confirm the reproducible results, the experiment was replicated three times. Sterile blank paper disks were impregnated with the solvent used as negative power. Antibiotic disks have also been used every time as a positive control.

3.5.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) were determined according to Doughari et al. (2007a and 2007b) with slide modification. The MIC of each extract was determined against each of the test bacteria in varying concentrations of 50, 75, 100, 125, 150, 175 mg/ml. Then the nutrient broth (9ml) tube was added with 1ml of test organism previously diluted to McFarland turbidity standards (OD=0.5). A tube containing only nutrient broth was seeded with the test bacteria, as described above, to serve as controls. MIC was measured by the concentration at which there was no change of turbidity according to Ajaiyeoba et al. (2003).

1 ML of bacterial culture was piped from a mixture obtained in the determination of MIC tubes that showed no growth and subcultured to nutrient agar and incubated for 24 hours at 37^0C. The concentration at which no single colony of bacteria was found to be taken as MBC after incubation.

3.6 Effect of neem leaf and papaya fruit extracts to control enterobacteria associated poultry diseases (*In vivo*)

This study was executed at the Mahbub poultry farm at Meherchondi, March 2016 to May 2016.

Preparation and management of Sonali chicken experimental shed

At first the experimental shed for the rearing of Sonali chicken was properly prepared. The experimental units were prepared on a floor litter system. Disinfect all the equipment by the purified water before putting the experimental chicks into the cages. Total 84 Sonali chicks (one day old) from a local hatchery were bought. These chicks have been put into the experimental cages and the chicks have been provided with the same conditions of management such as floor space, temperature, relative humidity, ventilation, and light. Vitamin-C and glucose were then given to the chicks to prevent

stress during transportation. This Sonali chicks were taken at the cages for 7 days in the same condition and preserved the temperature of brooding. During the first week, the chicks were brooded at 35^{o}C; the temperature was gradually reduced every week by 3^{o}C until the temperature reached an average, i.e., 25±1^{o}C. The handling of the litter was also done with great care. The Sonali chicken was properly supplied with the starter and grower Sonali rations. Twice a day, the birds were given a weighed sum of ration.

Collection of plant samples

Young and fresh neem leaves and papaya fruits were taken from the local area and market, respectively and brought to the laboratory.

Preparation of plant extract

Collected plant parts were washed with purified water properly and dried in air. Then the samples were ground separately with some distilled water and blend with a mixture blender. The extracts were filtered with Whatman no. 1 filter paper. These clear extracts were stored at 4^{0}C with an air-tight conical flask.

Application of plant extracts and inoculum on chick

After made the plant extracts, 50µl of each plant extract and an equal volume of inoculums were orally ingested to the 15-day old chicks separately after 4 days interval, up to 45 days.

The execution of the experiments

After 7 days of nursing all the chicks were selected for assessing the effect of neem leaf and papaya fruits extract on Sonali chick to control of entero-bacteria as the following treatments:

Used additive: Ciprofloxacin, Growth promoter, Vitamin and Saline.

Experiment

Group 1	**Group 2**
TNo (Control)	TPo (Control)
TN_1(Additive)	TP_1 (Additive)
TN_2 (Neem)	TP_2 (papaya)
TN_3 (Add.+Neem)	TP_3 (Add.+Papaya)
TN_4 (N+*Salmonella*)	TP_4 (P+*Salmonella*)
TN_5 (N+*Shigella*)	TP_5 (P+*Shigella*)
TN_6(N+*Enterobacter*)	TP_6 (P+*Enterobacter*)
TN_7 (N+*E.Coli*)	TP_7 (P+*E.Coli*)
TN_8(N+*Klebsiella*)	TP_8 (P+*Klebsiella*)
TN_9 (*Salmonella*)	TP_9 (*Salmonella*)
TN_{10} (*Shigella*)	TP_{10} (*Shigella*)
TN_{11} (*Klebsiella*)	TP_{11} (*Klebsiella*)
TN_{12} (*E.Coli*)	TTP_{12} (*E. Coli*)
TN_{13} (*Enterobacter*)	TP_{13} (*Enterobacter*)

These experimental chicks were closely observed for 15 to 45 days and the following steps were calculated.

Measurement of live weight: After 15^{th} days, 30 days, and 45 days the chicks were weighting separately for each treatment. The mean weight of each treated chicken was calculated.

Measurement of weight gain: Weight gain of each treated and control chickens were measured with the following formula:

$$\text{Weight gain (gm)} = \frac{\text{Live weight at particular days (gm)} - \text{initial weight (gm)}}{\text{Number of chicken used}}$$

Mortality: Mortality was recorded throughout the study.

Statistical Analysis

All experiments had at least three replications followed by a completely randomized block design (RBD) and the data were analyzed by DMRT with SPSS software version 12.

CHAPTER FOUR
RESULTS

4.1 Survey

In the present study total of twenty poultry farms of different places of Rajshahi Metropolis and surrounding areas were surveyed from January to December 2013. All of the farms were mainly large scale producer of poultry. They nourished mainly Sonali, broiler, layer and cock for meat and egg. The farms were mainly surveyed to assess their sanitation condition, the hygienic status of the personnel, and to analyze the rate of disease incidence of the poultry in different farms.

Survey data showed that different types of poultry (Sonali, Layer, Cock and Broiler) were raised for meat and egg production where specific poultry-feed (Table 3.1) was given to a particular type of poultry. Layer starter (age of 0-8 weeks), Layer grower (age of 9-20 weeks), Layer finisher (after 20 weeks to finish) was used for Sonali, Layer, and Cock, respectively whereas Broiler starter (age of 0-4 weeks), Broiler finisher (after 4 weeks to finish) was used for Broiler (Table 3.2).

Analysis of survey data on the disease incidence of poultry farms

From the survey record, it was observed that Coccidiosis, Avian influenza, Bacillary white diarrhea, Mycoplasmosis; Gambero, Ranikhet and Peritonitis (milky fluid) etc. were the most common poultry diseases in surveyed areas (data not shown). Diseases incidence of poultry flocks were significantly ($P \leq 0.5$) varied with the farms and poultry of different locations and the results are presented in Table 4.1. The highest DI (%) was recorded as 13.3, 10.99, 11.1, 7.29, 8.07, 7.09, 6.09, 7.07, 10.2, 8.22, 9.95 and 12.4 in January to December, respectively at Jewel poultry farm. Among the months the highest disease incidence (13.3%) was recorded in January. The moderate disease incidence was recorded in February; March, September and December where above 10% disease incidence was exhibited. The lowest disease incidence (0.0 to 6.09%) was recorded in July.

From the survey record, it was observed that the GID was significantly (P=0.5) varied with the month. The ranges of gastrointestinal diseases were recorded as 50.0 to 78.57% in January to December. The highest GID was recorded at 78.57% in January and the lowest was 50% in April (Fig 4.1).

Table 4.1 Disease incidence of poultry in different poultry farms in and around Rajshahi city during January to December, 2013.

Name of poultry farms	Types of flock	January DI(%)	February DI(%)	March DI(%)	April DI(%)	May DI(%)	June DI(%)	July DI(%)	August DI(%)	September DI(%)	October DI(%)	November DI(%)	December DI(%)
Ismail	Sonali	7.3de	4.76de	5.09cd	4.6de	3.05de	3.03ef	2.95ef	3.33de	4.09cd	5.4de	4.43de	4.3de
City	Sonali	7.1g	1.9gh	1.8g	2.0b	2.2ef	1.7hi	1.5hi	2.5ef	1.8e	3.1b	2.9gh	1.9g
Amar	Sonali	8.6b	7.5b	5.6bc	6.2h	5.3b	4.2b	3.8b	5.67b	4.6bc	7.23h	8.1b	7.5b
Rakib	Sonali	8.4fg	1.38c	2.1g	1.19c	2.2ef	3.2de	3.5de	2.41ef	1.1g	8.15c	1.48c	2.5fg
Mahbub	Sonali	6.82c	5.33h	5.08cd	5.05g	5.11b	1.36hi	1.46hi	5.51b	4.08cd	5.15g	5.43h	5.82c
Peyarul	Layer	6cd	5.0cd	4.09ef	1.21c	1.83ef	1.81hi	2.11hi	2.32ef	3.09ef	6.10cd	4.89cd	5.5cd
Juwel	Sonali	13.3a	10.99a	11.1a	7.29a	8.07a	7.09a	6.09a	7.07a	10.2a	8.22a	9.95a	12.4a
Ahadur	Cock	7.15cd	4.05ef	5.24cd	4.93f	4.06bc	1.66hi	2.06hi	4.11bc	4.24cd	5.91f	4.45ef	4.11cd
Belal	Layer	7.19fg	2.94gh	2.57fg	1.28c	0.59f	2.85fg	2.1hi	2.68f	1.57fg	4.15b	2.84gh	3.11fg
Ahab	Cock	8.6cd	4.66ef	4.74de	4.69ef	2.91de	2.66gh	2.46gh	3.12de	1.74de	5.64ef	4.56ef	5.1cd
Iffat	Sonali	7.16gf	3.3fg	3.05fg	3.01fg	2.07ef	2.09gh	2.29gh	2.23ef	2.05fg	4.11fg	3.39fg	2.96gf
Sakil	Layer	7.8cd	4.76de	4.4de	4.6fg	5.1b	4.3b	5.3b	4.99b	3.4de	5.56fg	4.56de	4.9cd
Sojib	Sonali	6.0de	4.07ef	3.2fg	4.3de	3.1de	4.1bc	5.1bc	3.2de	2.2fg	5.33de	4.14ef	5.0de
Bulbul	Sonali	8.8de	3.2fg	3.1fg	3.05b	3.1de	2.9fg	3.1fg	2.98de	2.1fg	4.15b	3.21fg	4.7de
Masum	Broilar	9.33b	8.33b	7.2b	5.0cd	3.50cd	3.09cd	2.99cd	3.49cd	6.2b	6.10cd	8.21b	8.9b
Khokon	Cock	6.4c	4.9cd	5.17cd	5.03e	1.74ef	2.75fg	2.75fg	1.85ef	4.17cd	6.103e	4.85cd	6.7c
Rosid .	Cock	7.59ef	3.22fg	3.1fg	3.57fg	2.0f	0.66j	0.46j	2.1f	0.0h	4.51fg	3.31fg	4.79ef
Mostak	Layer	7.2g	1.23h	4.97de	2.13h	0.54f	1.62hi	1.63hi	0.54f	3.97de	3.11h	2.0h	3.1g
Sohidul	Sonali	8.9fg	2.82gh	2.07fg	2.8gh	2.03ef	2.01gh	2.29gh	2.03ef	1.07fg	3.81gh	2.72gh	3.5fg
Saon	Sonali	7.8fg	2.97gh	2.01g	1.9g	1.85ef	1.95hi	1.85hi	1.85ef	1.01g	2.92g	2.89gh	3.0fg

[Values in a column having same letter did not differ significantly P= 0.05 according to DMRT]

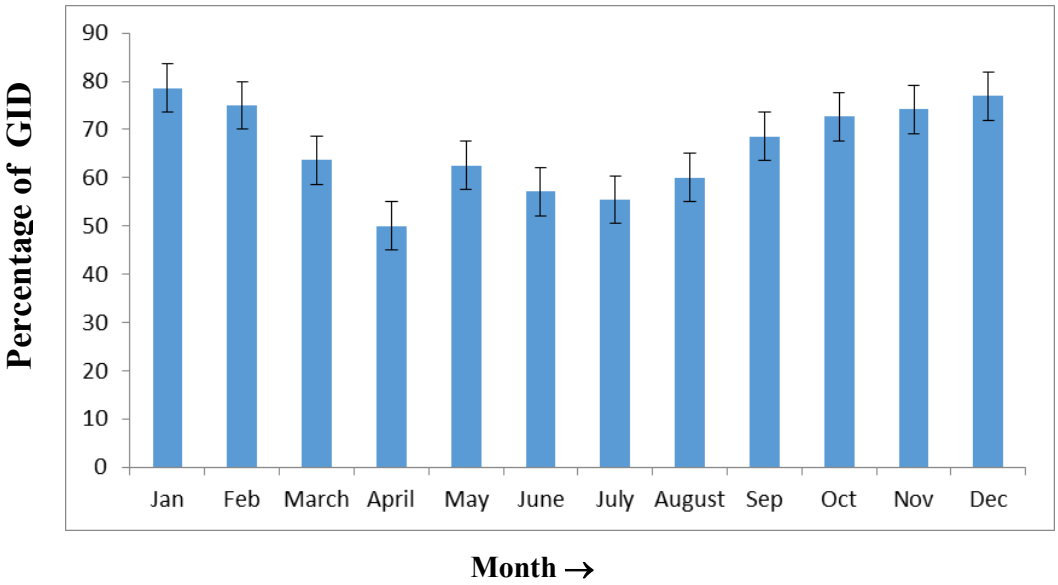

Fig 4.1 Comparison of percentage of gastro-intestinal diseases (GID) in surveyed area during January to December, 2013

Plate 4.1 Photographs showing gastro-intestinal disease symptom

Analysis of temperature and relative humidity (%) of poultry farms and their relationship with disease incidence

Temperature and relative humidity (%) of each poultry farm were recorded digitally from January to December 2013 and their relationships with disease incidence (DI) are presented in Fig. 4.2-4.13. The ranges of temperature and RH (%) were recorded as 15-17ºC and 76-79.2% in January while DI was 7.1 to 13.3%; 18.0-20.2ºC and 72.0-76.9% in February while DI was 1.23 to 10.99%; 27.0-29.5ºC and 64.0-68.2% in March while DI was 1.8 to 1.1%; 32.0-35.0ºC and 65.0-68.2% in April while DI was 1.1 to 7.29%; 34.0-36.9ºC and 79.0-82.5% in May while DI was 1.74 to 8.07%; 35.0-36.2ºC and 82.0-84.2% in June while DI was 1.36 to 7.09%; 27.8-31.2 ºC and 82.3-88.0% in July while DI was 1.46 to 6.09%; 27.8-31.5 ºC and 86.0-88.9% in August while DI was 1.85 to 7.07%; 27.0-32.3 ºC and 84.0-87.1% in September while DI was 1.1 to 10.2%; 26.0-28.9ºC and 85.0-88.8% in October while DI was 2.92 to 8.22%; 21.1-28.7ºC and 75.2-79.1% in November while DI was 1.48 to 9.95% and 17.8-20.5ºC and 77.5-86.3% in December while DI was 1.9 to 12.4%, respectively. From the results, it was observed that the disease incidence was directly correlated with temperature, not RH.

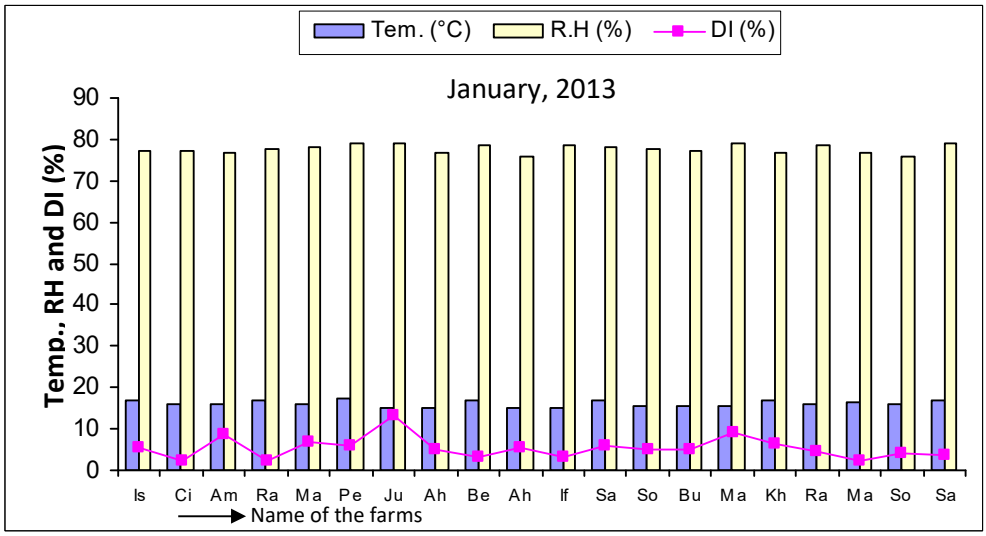

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Fig 4.2 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

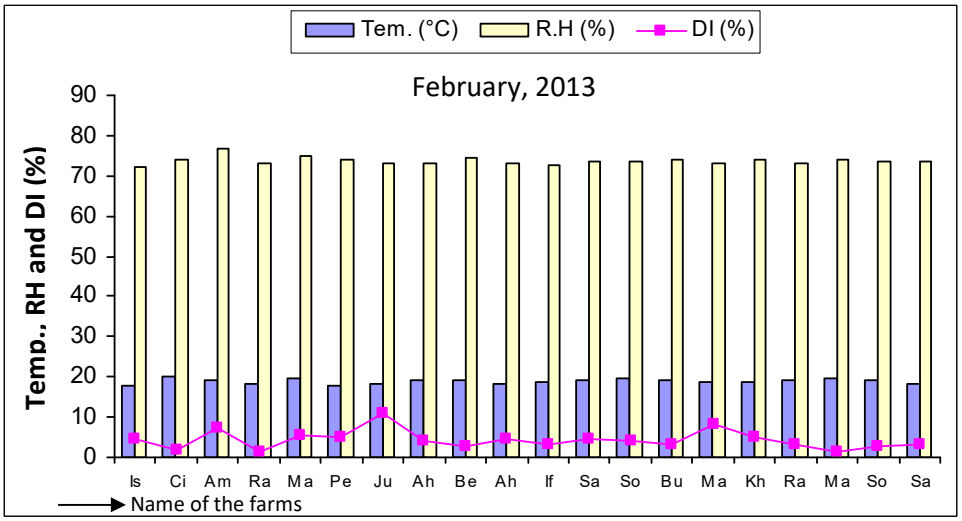

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Fig 4.3 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

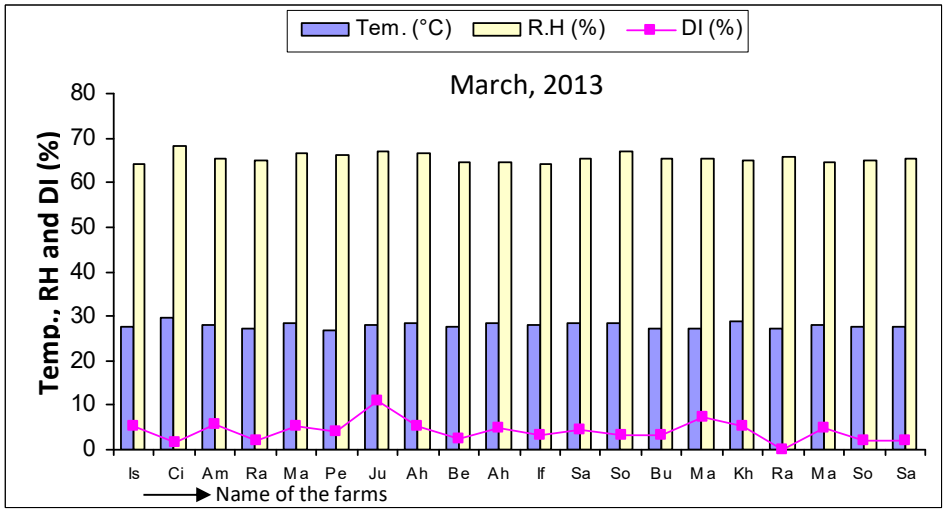

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Fig 4.4 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

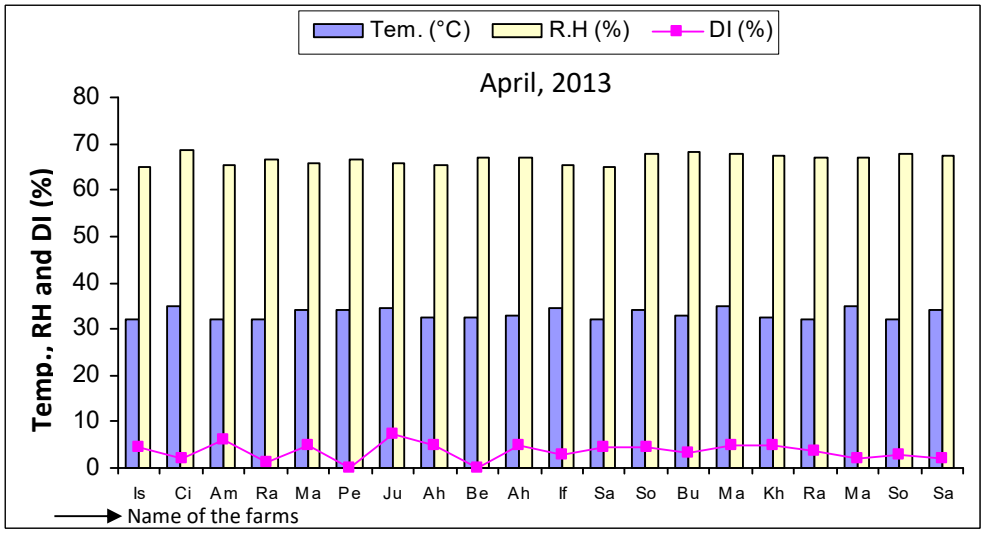

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Fig 4.5 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

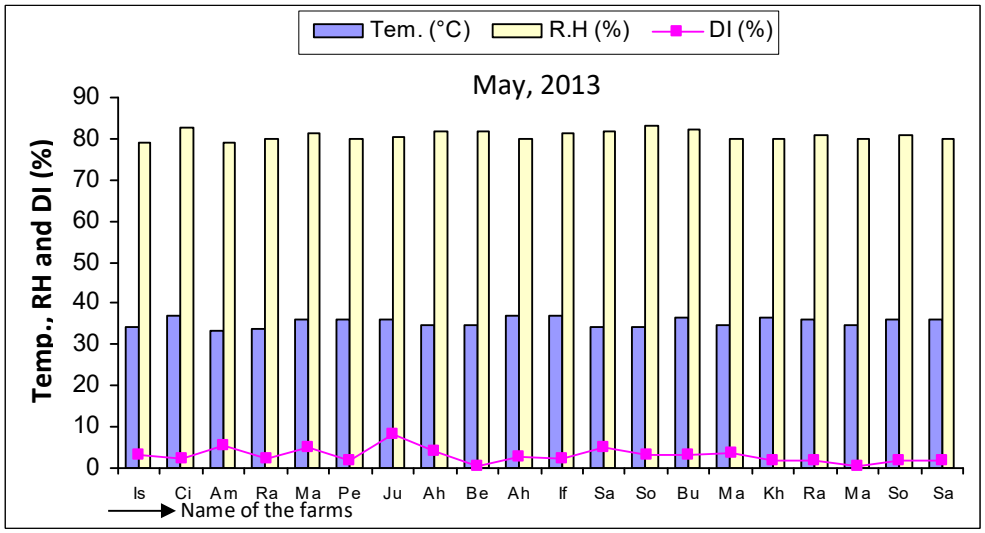

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Fig 4.6 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

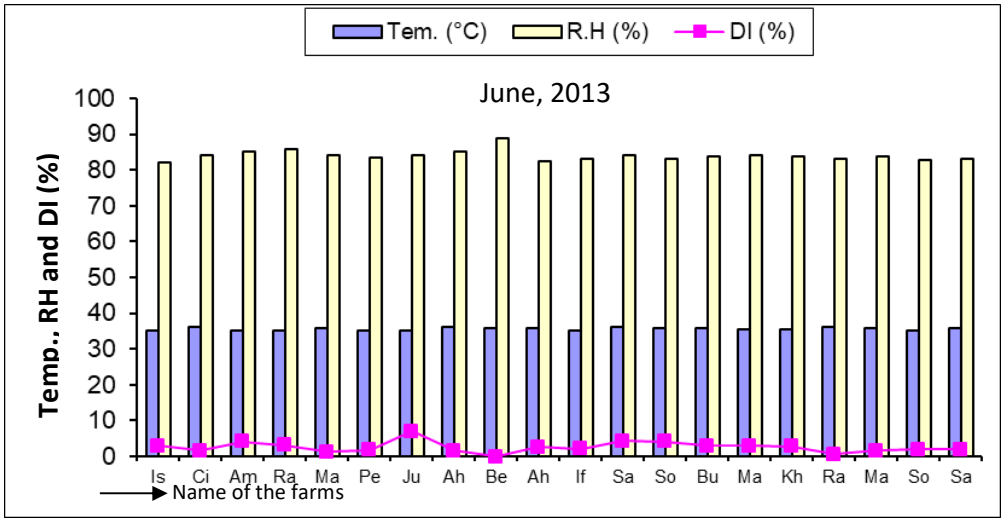

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Fig 4.7 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

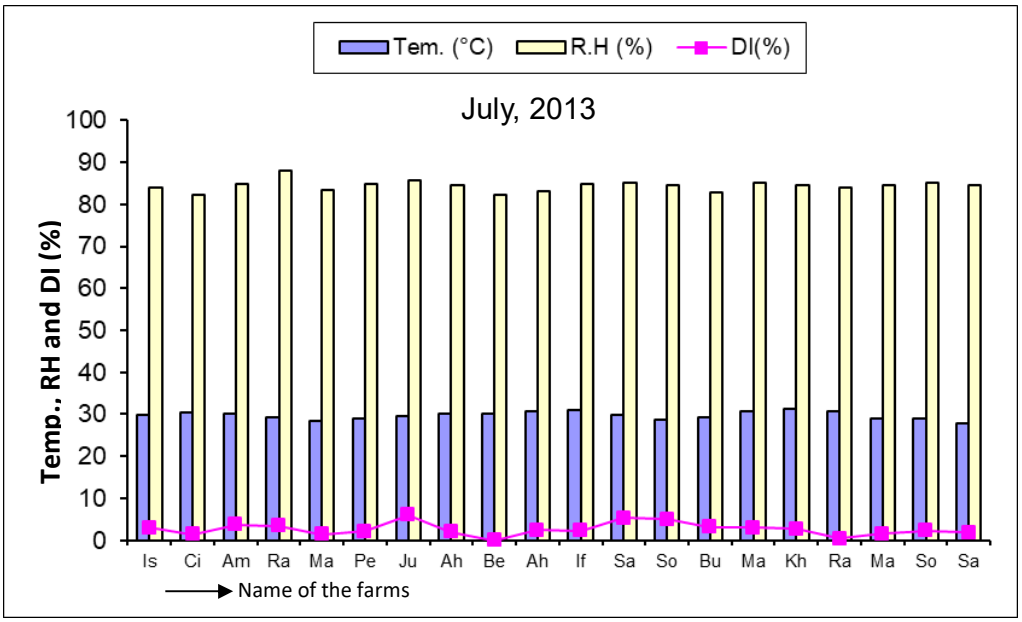

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Fig 4.8 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

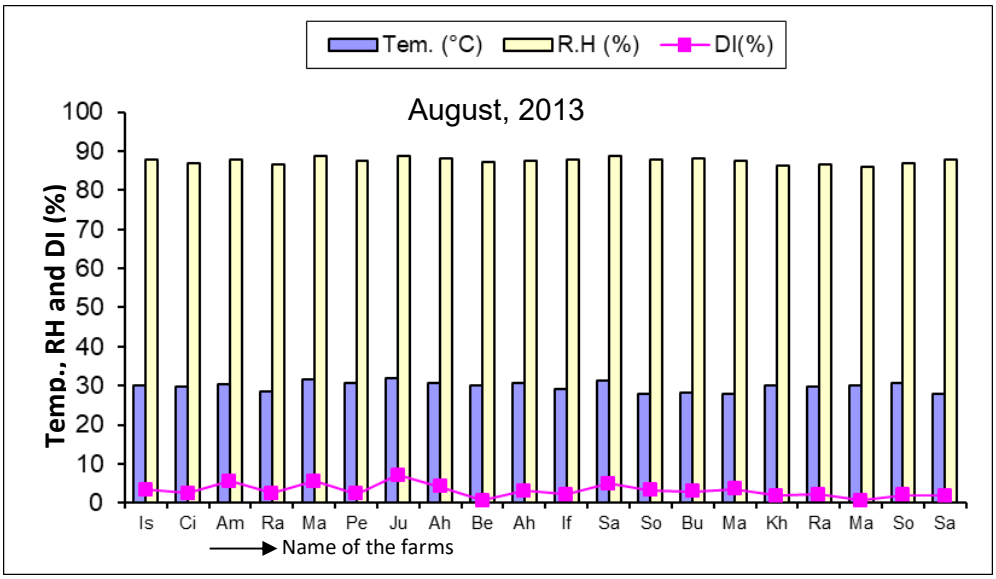

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Fig 4.9 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

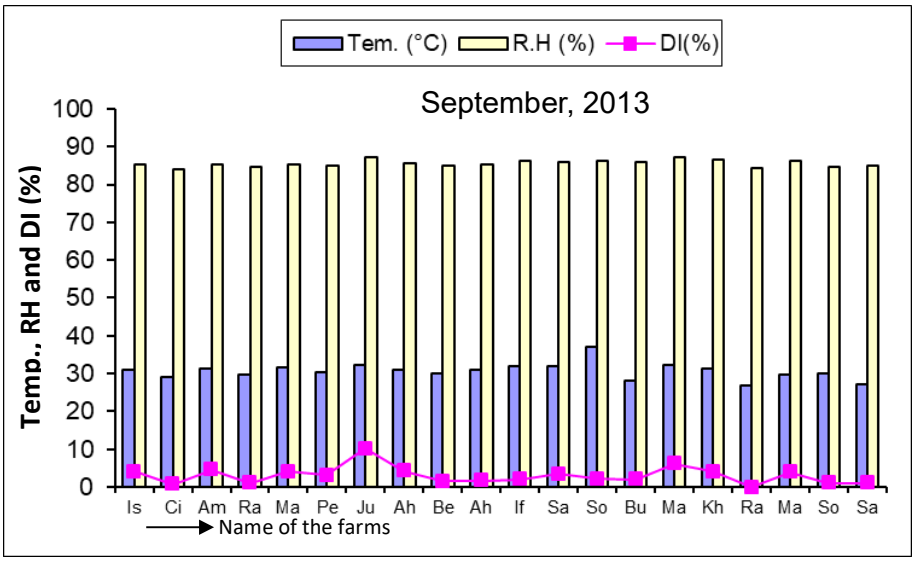

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Fig 4.10 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

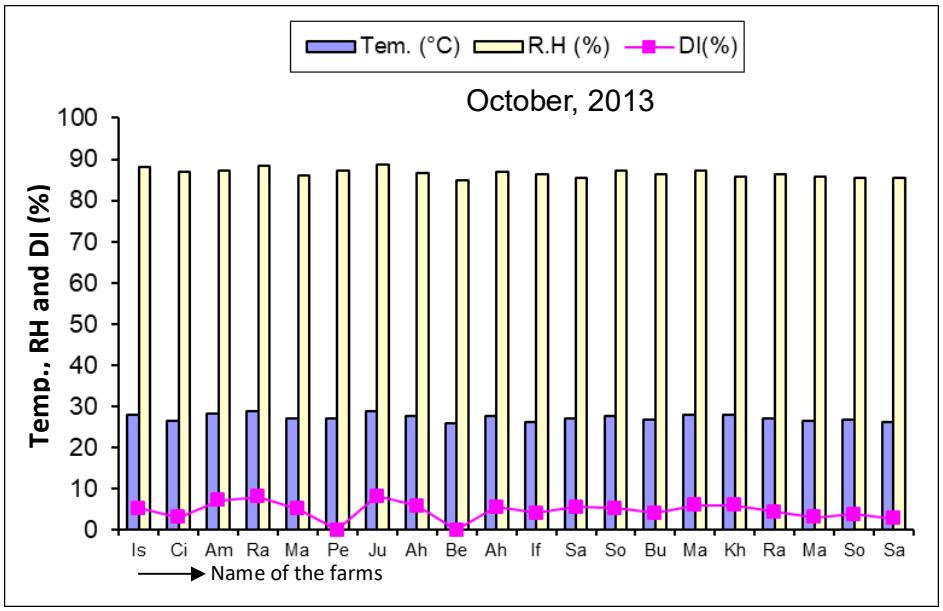

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Fig 4.11 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

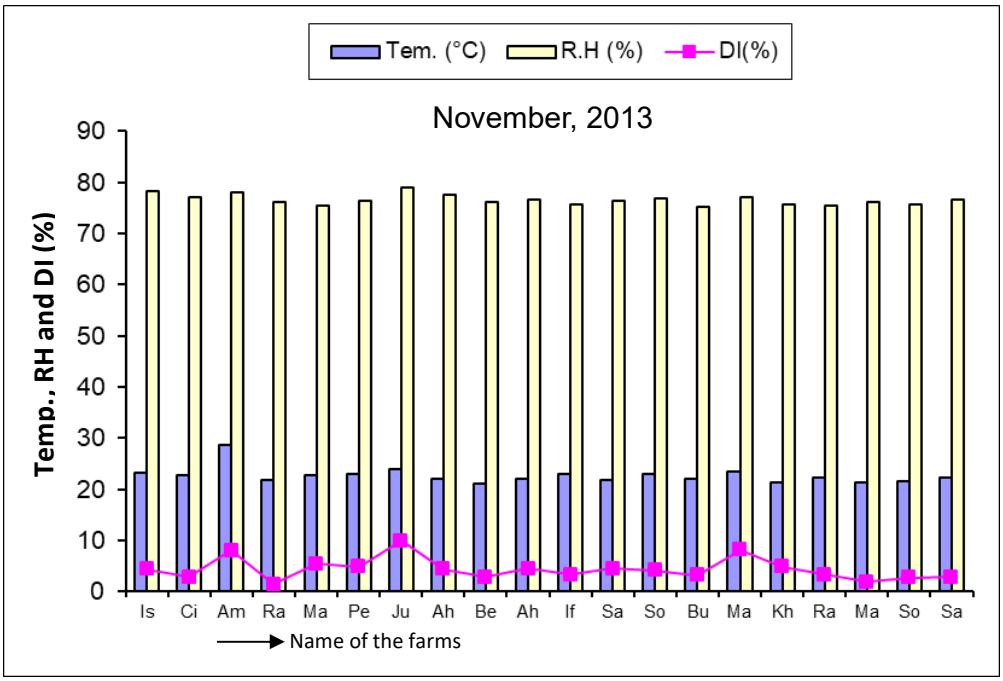

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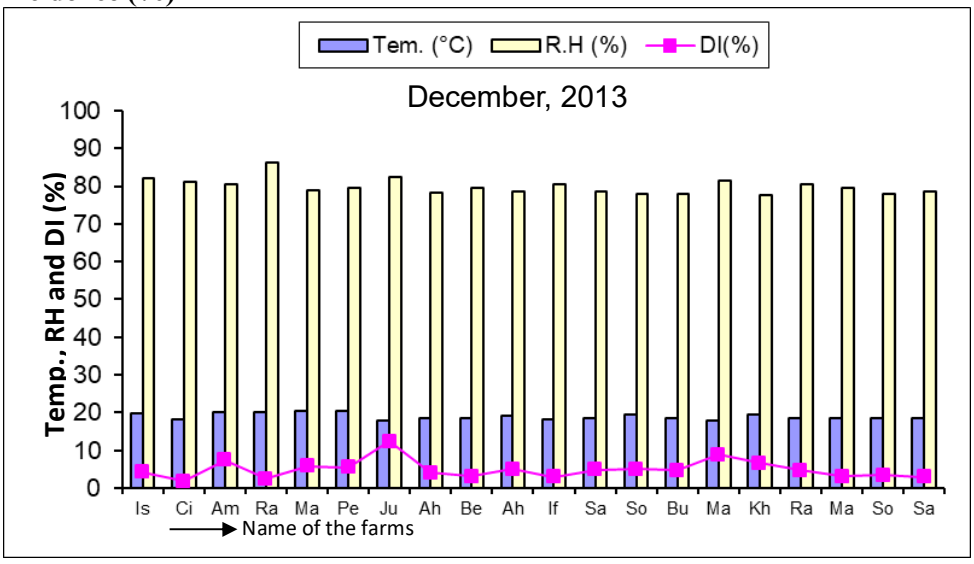
Fig 4.12 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)



[Here,Is=Ismail, Ci=City, Am=amar, Ra=Rakib, Ma=Mahbub, Pe=Peyarul, Ju=Juwel, Ah=Ahab, Be=Belal, Ah=Ahadur, If=Iffat, Sa=Sakil, So=Sojib, Bu=Bulbul, Ma=Masum, So=Sohidul, Sa=Saon.]

Fig 4.13 Relationship of temperature (°C) and relative humidity (%) with disease incidence (%)

Analysis of survey data on hygiene and sanitation status of the farms

The hygiene and sanitation status of the poultry farms were surveyed and the results are presented in Table 4.2. The statuses of the cleanness of all the farms were good. They cleaned the farms regularly. For sanitary practices, they used timsen, glutex, lifeline etc as sanitizer (not shown in table). Among the farms, 55% (n=11/20) percent of farms used sanitizer regularly were 45% (n=9/20) percent of farms were irregular to use sanitizer. The farms used to supply water, tube well water and deep tube well water to drink poultry. Out of twenty farms, 60% (n=12/20) were used tube-well water, 20% (n=4/20) percent used to supply water and deep tube-well water 20% (n=4/20). In-room condition, excellent light, ventilation and dampness was observed in 45% (n=9/20), 70% (n=14/20) and 60% (n=12/20), respectively and rest of the farms were in good conditions

Table 4.2 Hygiene and sanitation status and room condition of surveyed farms.

Name of area	Types of flock	Hygiene & sanitation status			Room condition		
		Cleanliness	Used sanitizer	Water source	Light	Ventilation	Dampness
Ismail	Sonali	+	Regularly	Supply	+ + +	+++	+++
City	Sonali	+	Regularly	Supply	+ + +	+++	+++
Amar	Sonali	+	Irregularly	Supply	+ +	+++	++
Rakib	Sonali	+	Regularly	Supply	+ + +	+++	+++
Mahbub	Sonali	+	Irregularly	Tubewell	+ +	++	++
Peyarul	Layer	+	Regularly	Tubewell	+ + +	+++	+++
Juwel	Sonali	-	Irregularly	Tubewell	+ +	++	+
Ahadur	Cock	+	Irregularly	Tubewell	+ +	++	++
Belal	Layer	+	Regularly	Tubewell	+ + +	+++	+++
Ahab	Cock	+	Irregularly	Tubewell	+ +	++	+++
Iffat	Sonali	+	Regularly	Tubewell	+ + +	+++	+++
Sakil	Layer	+	Irregularly	Tubewell	+ +	++	++
Sojib	Sonali	+	Regularly	Tubewell	+ + +	+++	+++
Bulbul	Sonali	+	Regularly	Tubewell	+ + +	+++	+++
Masum	Broilar	+	Irregularly	Tubewell	+ +	++	++
Khokon	Cock	+	Irregularly	Tubewell	+ +	+++	++
Rosid .	Cock	+	Irregularly	Deep Tub.	+ +	+++	++
Mostak	Layer	+	Regularly	Deep Tub.	+ + +	+++	+++
Sohidul	Sonali	+	Regularly	Deep Tub.	+ + +	+++	+++
Saon	Sonali	+	Regularly	Deep Tub.	+ + +	+++	+++

[Here, - = Poor, + = Good, ++ = Better, +++ = Excellent]

Analysis of survey data on hygiene practices, sanitary practices and satisfactory level of the farms

From survey data, it was observed that the poultry workers were not aware of their personal hygiene. They did not use footbaths, gloves, hairnets, and boots while coming into and out of the pens. From Table 4.3 it was observed that 15% (n=3/20) of the poultry workers scored 'excellent' whereas 15% (n=3/20) poultry farm workers scored 'very good', 55% (n=11/20) workers scored 'good' and15% (n=3/20) scored the least level of hygiene. It was also observed that the workers used glutex spray on foot while they entered into the pens and they also washed their hands after coming from the pens. 30% (n=6/20) of poultry farms scored excellent in case of hygiene practices of pens whereas 60% (n=12/20) farms scored 'very good' and 10% (n=2/20) farms scored 'Good' in the scale of sanitary condition.

From Table 4.3 it was observed that the waste management system (liquid and solid litter) of the poultry farms in Rajshahi city was not appropriate (Table 4.3 and plate 4.2). In the case of liquid litter disposal, 20% (n=4/20) of poultry farms did not follow any waste disposal system whereas 55% (n=11/20) farms scored 'Good' in liquid disposal while 25% (n=5/20) farms scored 'very good' in liquid disposal (Table 4.3). All poultry farms scored 'very good' in solid litter disposal. From the survey record, it was also observed that there was no variation in the case of a solid litter disposal system. Overall it was observed that the practice of deposing litter for several days was followed in most of the farms. Later on, decomposed waste materials were used as feed for pisciculture and sometimes it was used as fertilizer in agricultural land. No septic tank was found. The absorption pit was not constructed to treat daily sewage like sweeping of poultry sheds to remove litter or used chemicals or sanitizers during cleaning.

The satisfactory level of the poultry farms concerning hygiene and sanitary practices were analyzed

Of the twenty poultry farms surveyed, 15% (n=3/20) of farms scored 'satisfactory" to their hygienic status and sanitary practices cumulatively (according to European Poultry Meat Industry Guide, February-2010). However, in scale regarding the parameters of hygienic status and sanitary practices, the rest of 85% (n=17/20) farms scored 'not satisfactory' (Table 4.3).

Table 4.3 Hygiene practices, sanitary practices and satisfactory level of the poultry farms and workers

Name of farms & location	Hygiene practices		Sanitary practices		Satisfactory scale
	Personnel	Pen	Liquid litter	Solid litter	
Ismail, Court	+	++	-	++	NS
City, Nogarpara east	++	+++	+	++	NS
Amar, Nogarpara North	-	+	-	++	NS
Rakib, Daingpara	++	+++	++	++	NS
Mahbub, Mershondi	+	++	+	++	NS
Peyarul, Khorkhory	+++	+++	++	++	S
Juwel, Chokpara	-	+	-	++	NS
Ahadur, Narikelbaria	+	++	++	++	NS
Belal, Noudapara	+++	+++	++	++	S
Ahab, baya	+	++	+	++	NS
Iffat, Dangipara	++	++	-	++	NS
Sakil, Parisal	+	++	+	++	NS
Sojib, Horipur	+	++	+	++	NS
Bulbul, Haragram	+	+++	+	++	NS
Masum, Darusa	+	++	+	++	NS
Khokon, Chormajardiar	-	++	+	++	NS
Rosid, Budpara	+	++	+	++	NS
Mostak, Dasmari	+++	+++	++	++	S
Sohidul, Katakhali	+	++	+	++	NS
Saon, Horian	+	++	+	++	NS

[- = Poor; + =Good; ++ =Better; +++ =Excellent; S=Satisfactory; NS=Not Satisfactory]

Plate 4.2 Photographs showing liquid litter management system (A) & (B) and solid litter management system (C) & (D) in a poultry farm.

Comparison of Disease Incidence (%) with water sources and hygiene practices

To determine the relationship of DI (%) with water sources and hygiene practices the survey data (Table 4.4) were analyzed and the summarized results are presented in Fig 4.14 to 4.16. Among the flocks, broiler were more sensitive to be infected by disease agent (mean DI= 6.92%) rather than other Cock (mean DI= 3.74%), Sonali (mean DI= 3.18%) and Layer (mean DI= 2.89%) (Table 4.4 and Fig 4.14). Minimum disease incidence was found in those poultry farms where deep tube-well water (mean DI= 2.4%) was used rather than supply water (mean DI= 3.76%) and tube-well water user (mean DI= 4.28%), (Fig 4.15). On the other hand disease incidences (%) of the flocks were directly correlated to personal hygiene and DI (%) was gradually reduced where excellent personal hygiene practices were maintained (Fig 4.16).

Table 4.4 Relationship of disease incidence with flocks, water sources, and hygiene practices with

Name of area	Types of flock	Water source	Hygiene practices	Mean disease incidence (%)
Ismail	Sonali	Supply	+	4.19
City	Sonali	Supply	+++	2.03
Amar	Sonali	Supply	-	6.19
Rakib	Sonali	Supply	++	2.63
Mahbub	Sonali	Tubewell	+	4.68
Peyarul	Layer	Tubewell	+++	3.05
Juwel	Sonali	Tubewell	-	8.4
Ahadur	Cock	Tubewell	+	4.16
Belal	Layer	Tubewell	+++	1.46
Ahab	Cock	Tubewell	+	3.99
Iffat	Sonali	Tubewell	+++	2.81
Sakil	Layer	Tubewell	+	4.81
Sojib	Sonali	Tubewell	-	4.06
Bulbul	Sonali	Tubewell	+	3.37
Masum	Broilar	Tubewell	+	6.93
Khokon	Cock	Tubewell	-	4.37
Rosid .	Cock	Deep Tub.	++	2.43
Mostak	Layer	Deep Tub.	+++	2.25
Sohidul	Sonali	Deep Tub.	+++	2.59
Saon	Sonali	Deep Tub.	++	2.33

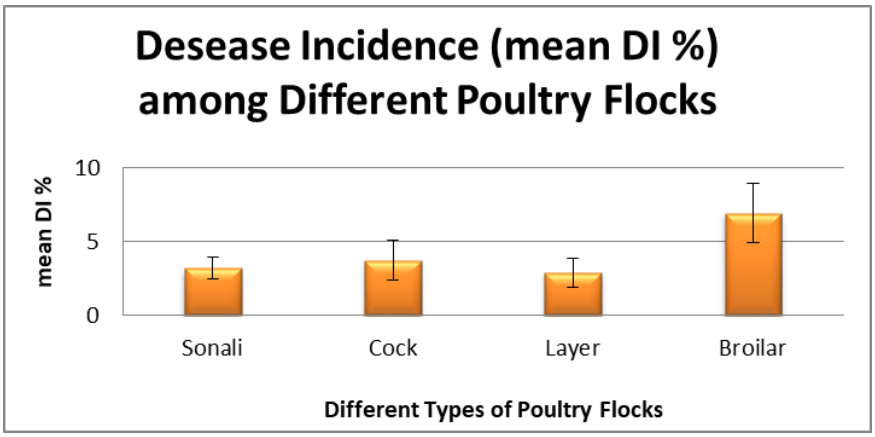


Fig 4.14 Relationship of disease incidence (DI) with types of flock

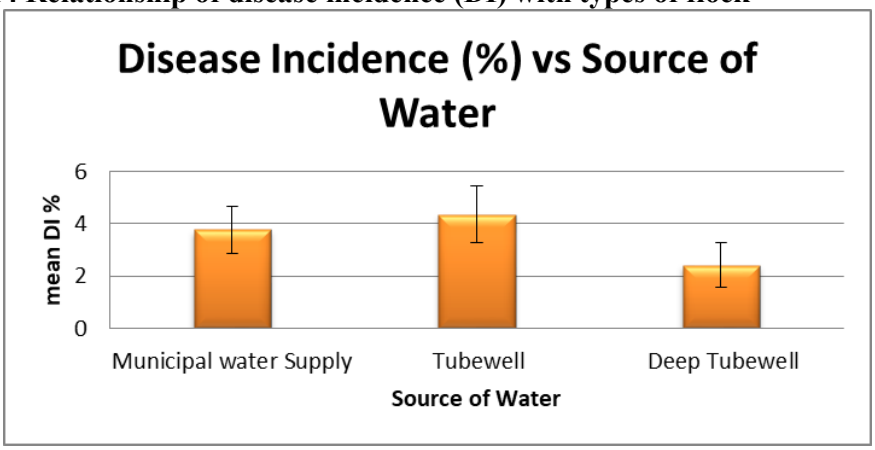


Fig 4.15 Relationship of of disease inidence (%) in poultry flocks with water sources.

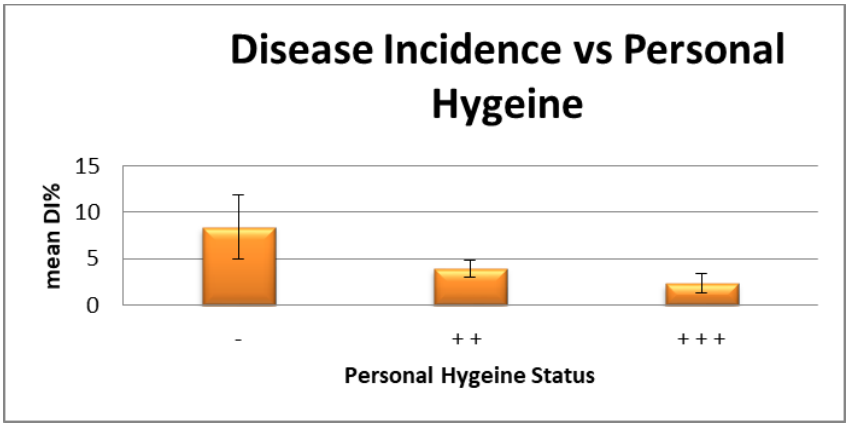


Fig 4.16 Relationship of disease incidence (%) in poultry flocks with personal hygiene status of the poultry workers.

Recommendation

Sanitation and hygiene practices of different poultry farms and personnel of Rajshahi city were surveyed. Based on survey results some exclusive and key points were including as recommendation for fruitful poultry farming as below:

i. The poultry workers should wear footwear, hairnet, gloves etc. during the rearing of poultry.
ii. They should use sanitizer properly at the time of entry and exit of the pens of the farms.
iii. Poultry should border in intensive temperature and relative humidity.
iv. For improvement of sanitation practices, each poultry farm should build a septic tank.
v. For proper solid waste management, farmers should use covered bins for build compost pit.
vi. About improve hygiene status regular monitoring of poultry farms and feeds should be needed.
vii. Farmers should be encouraged to maintain healthy practices and print and electronic media can contribute to it.
viii. The government should provide national and regional policies and guidelines for healthy farming.
ix. Finally, integration of farmer awareness, proper guidelines, and a strong monitoring system can make a successful farming system in the future.

4.2 Microbial quality assessment of poultry feeds

In the present study, poultry feeds samples were collected from the different poultry farms of Rajshahi Metropolis and surrounding areas from January to December 2013. These feeds were subjected to microbial quality assessment. For this purpose, Nutrient agar (NA), MacConkey, Ethylene methyl blue (EMB), Salmonella-Shigella (SS) media were used to analyze of microbial safety of the feeds and the results are presented below:

Total aerobic plate count (APC) of poultry feeds samples

Poultry feeds samples were plated on NA medium and the colony-forming unit (cfu) of total viable bacteria were counted (Table 4.5 and Fig 4.3). The ranges of cfu for APC were recorded as 0.28×10^4 to 2.8×10^8/gm. The highest cfu was counted as 2.8×10^8/gm at Juwel poultry farm and the lowest was 0.28×10^4/gm at Ismail poultry farm. Among the poultry farms, 33.33 - 83.33% of samples showed $> 10^6$ cfu/g.

The total coliform count of poultry feeds samples on MacConkey agar

MacConkey agar medium was used for the total coliform count. The samples were plated on MacConkey medium and the cfu were counted (Table 4.5 and Fig 4.3). The ranges of cfu of total coliforms were counted as 0.1×10^4 to 9.6×10^4/gm. The highest cfu was counted 9.6×10^5/gm at Peyarul poultry farm and the lowest was 0.1×10^4/gm at Mostak poultry farm.

Total *Escherichia coli* count of poultry feed samples on EMB agar medium

Eosin methylene blue (EMB) medium was used for *E. coli* count. The samples were plated on EMB medium and the cfu were counted (Table 4.5 and Fig 4.3). The ranges of cfu of *E. coli* were counted as 0.0 to 1.80×10^4/gm. The highest cfu was counted as 1.80×10^4/gm at Juwel poultry farm. *E. coli* were not detected at Roshid and Ahab poultry farms.

Total *Salmonella* sp. and *Shigella* sp. count of poultry feeds samples on SS agar medium

Salmonella-Shigella (SS) agar medium was used for *total Salmonella* count. The samples were plated on EMB medium and the cfu were counted (Table 4.5 and Fig 4.3). The ranges of cfu of total Salmonella were counted as 0.0 to 0.85×10^4/gm. The highest cfu was counted 0.85×10^4/gm at Juwel poultry farm and the lowest was counted as 0.0/gm at Peyarul and Mostak poultry farms.

Table 4.5 Ranges of cfu/gm of poultry feed samples on different media

Location	Ranges of cfu/gm			
	NA	MacConkey	EMB	SS
Ismail Poultry Farm, Court	$0.28\times10^4$to 1.4×10^7	1.2×10^4 to 6.5×10^4	$0.03\times10^4$to 0.48×10^4	$0.02\times10^4$to 0.80×10^4
Mahbub Poultry Farm, Meherchondi	1.1×10^6 to 2.3×10^8	0.4×10^4 to 5.2×10^4	$0.04\times10^4$to 0.96×10^4	2.26×10^3 to 4.13×10^3
Peyarul poulty farm, Khorkhory	1.9×10^4 to 2.2×10^7	$0.8\times10^4$to 9.6×10^4	$0.10\times10^4$to 0.52×10^4	0.0 to 0.31×10^4
Juwel Poultry Farm, Chokpara	1.6×10^5 to 2.8×10^8	0.8×10^4 to 9.5×10^4	$0.16\times10^4$to 1.80×10^4	$0.03\times10^4$to 0.85×10^4
Ahadur poultry farm, Narikelbaria	$1.6\times10^5$to 2.8×10^8	$0.3\times10^4$to 2.4×10^4	$0.05\times10^4$to 1.12×10^4	$0.07\times10^4$to 0.22×10^4
Khokon Poultry Farm, Chor maajardiar	1.8×10^4 to 1.2×10^8	0.3×10^4 to 2.5×10^4	$0.17\times10^4$to 1.12×10^4	$0.09\times10^4$to 0.54×10^4
Rosid Poultry Farm, Maherchondi	$1.2\times10^4$to 1.6×10^8	1.1×10^4 to 2.9×10^4	0.0 to 1.36×10^4	$0.11\times10^4$to 0.27×10^4
Mostak Poultry Farm, Dasmari	1.7×10^4 to 1.8×10^8	0.1×10^4 to 2.4×10^4	0.02×10^4 to 0.65×10^4	0.0 to 0.25×10^4
Belal Poultry Farm, Noudapara	$1.7\times10^5$to 2.4×10^7	0.6×10^4 to 8.5×10^4	$0.26\times10^4$to 0.76×10^4	$0.11\times10^4$to 0.20×10^4
Ahab poultry farm, Baya	$1.3\times10^5$to 1.4×10^8	$0.3\times10^4$to 2.3×10^4	0.0 to 0.60×10^4	$0.08\times10^4$to 0.19×10^4
Iffat Poultry Farm, Dangipara	1.2×10^4 to 1.6×10^8	0.3×10^4 to 2.5×10^4	$0.08\times10^4$to 0.62×10^4	$0.05\times10^4$to 0.20×10^4
Sakil Poultry Farm, Parisal	1.7×10^4 to 1.8×10^8	0.4×10^4 to 6.5×10	$0.09\times10^4$to 0.70×10^4	$0.02\times10^4$to 0.16×10^4
City poultry farm, Ngorpara	$0.45\times10^5$to 2.3×10^8	$0.3\times10^4$to 2.9×10^4	$0.05\times10^4$to 0.07×10^4	$0.03\times10^4$to 0.15×10^4
Amar poultry farm, Nogorpara	$1.2\times10^4$to 1.6×10^8	$0.3\times10^4$to 6.5×10^4	$0.05\times10^4$to 0.46×10^4	$0.09\times10^4$to 0.54×10^4
Rakib Poultry Farm, Daingpara	1.6×10^6 to 2.3×10^8	0.6×10^4 to 2.5×10^4	$0.8\times10^4$to 0.60×10^4	$0.9\times10^4$to 0.54×10^4
Sohidul Poultry Farm, Katakhali	$1.7\times10^4$to 1.8×10^8	1.7×10^4 to 6.4×10^4	$0.06\times10^4$to 0.76×10^4	$0.08\times10^4$to 0.20×10^4
Sojib Poultry Farm, Horipur	$1.6\times10^4$to 2.3×10^8	0.3×10^4 to 2.9×10	$0.11\times10^4$to 0.27×10^4	$0.11\times10^4$to 0.27×10^4
Bulbul Poultry Farm, Haragram	$1.6\times10^4$to 2.3×10^8	0.3×10^4 to 2.5×10^4	$0.14\times10^4$to 0.21×10^4	$0.14\times10^4$to 0.21×10^4
Saon poultry farm, Horian	$2.0\times10^4$to 2.1×10^7	$0.3\times10^4$to 2.1×10^4	$0.1\times10^4$to 0.8×10^4	$0.1\times10^4$to 0.8×10^4
Masum Poultry Farm, Darusa	1.9×10^6 to 1.4×10^8	0.6×10^4 to 6.9×10^4	$0.08\times10^4$to 0.19×10^4	$0.08\times10^4$to 0.19×10^4

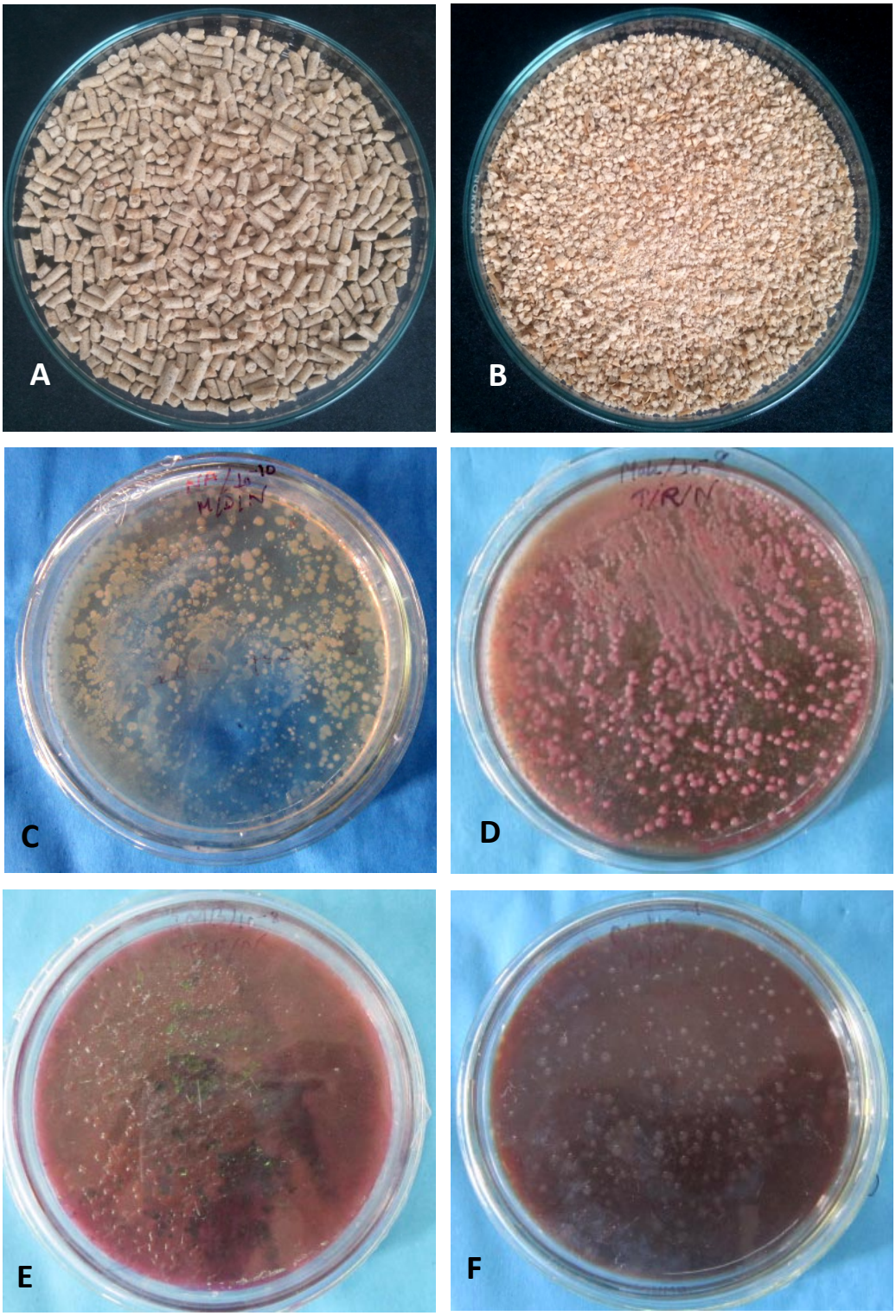


Plate 4.3 Photographs showing poultry feeds and bacterial colonies on a different medium: Poultry feeds (A, B); bacterial colonies on NA agar (C), MacConkey agar (D), EMB agar (E) and SS agar (F) plate.

4.3 Characterizations and identification of the pathogenic enterobacteria

4.3.1 Isolation of enterobacteria from poultry feeds

For isolation of enterobacteria feed three selective mediums namely MacConkey agar, EMB agar and SS agar were used. The feed samples were plated on these mediums separately for 24 hours. After that, the morphologically distinct bacterial colonies were removed and transfer into a vial. These isolates were coded with their sources.

Characterizations of colonies of the isolates

The colony morphology of the isolates on three selective medium MacConkey agar, EMB agar and SS agar were observed and their distinct features were studied according to their form, color, elevation, margin, and surface and the results are presented in Table 4.6 to 4.8.

Table 4.6 Cultural characteristics of the isolates on MacConkey agar plate

Isolate code	Culture characteristics on MacConkey agar				
	Form	**Color**	**Elevation**	**Margin**	**Surface**
MSM-30, IGM-32, JSN-40	Shiny	Creamy	Raised	Entire	Smooth
RGM-25, AGM-26, MLM-28, RSM-34, ISM-44, ,PLM-42	Circular	Creamy	Raised	Wavy	Rough
PLM-27, JSM-29, KSM-31, JGM-33, MGM-41, MSM-43 ISM-44	Shiny	White	Raised	Lobate	Smooth

Table 4.7 Cultural characteristics of the isolates on EMB agar plate

Isolate code	Culture characteristics on EMB agar				
	Form	**Color**	**Elevation**	**Margin**	**Surface**
SGE-1, MSE-5, SSE-6,PLE-7, AGE-37 JSE-36,SGE-39	Shiny	Colorless	Flat	Wavy	Smooth
JSE-2, ISE-4, JGE-8, BSE-38	Shiny	Green Metalic	convex	Entire	Smooth
MLE-3, MJE-9, IGE-10, SGE-11, MGE-12, RGE-35	Irregular	Colorless	Convex	Entire	Rough

Table 4.8 Cultural characteristics of the isolates on SS agar plate

Isolates Code	Culture characteristics on SS agar				
	Form	**Color**	**Elevation**	**Margin**	**Surface**
RGS-13, SGS-16, PLS-19, RGS-20, AGS-21, JGS-22, RSS-23, MSS-24	Circular	Colorless	Raised	Wavy	Smooth
JSS-14, ASS-15, MGS-17, MLS-18	Shiny	Colorless	Entire	Wavy	Smooth

Microscopic features of the isolates

After Gram staining, bacterial cells were observed under a microscope and their characteristics were recorded which are presented in Table 4.9 and Plate 4.4. The shapes of the bacterial isolates were long rod, moderate rod and short rod. All the isolates showed negative Gram staining reactivity.

Table 4.9 Microscopic features of the isolates

Isolate code	Shape of vegetative cells	Gram Staining Reaction
RGS-13, JSS-14, ASS-15, SGS-16, MGS-17, MLS-18, PLS-19, RGS-20, AGS-21, JGS-22, RSS-23, MSS-24	Long rod	-
RGM-25, AGM-26, PLM-27,MLM-28 JSM-29, MSM-30, KSM-31, IGM-32, JGM-33, RSM-34, BSE-38, JSM-40	Moderate rod	-
RGS-13, JSS-14, ASS-15, SGS-16, MGS-17, MLS-18, PLS-19, RGS-20, AGS-21, JGS-22, RSS-23, MSS-24	Short rod	-
RGE-35, JSE-36, AGE-37, SGE-39, MGM-41, PLM-42, MSM-43, ISM-44	Short rod	-

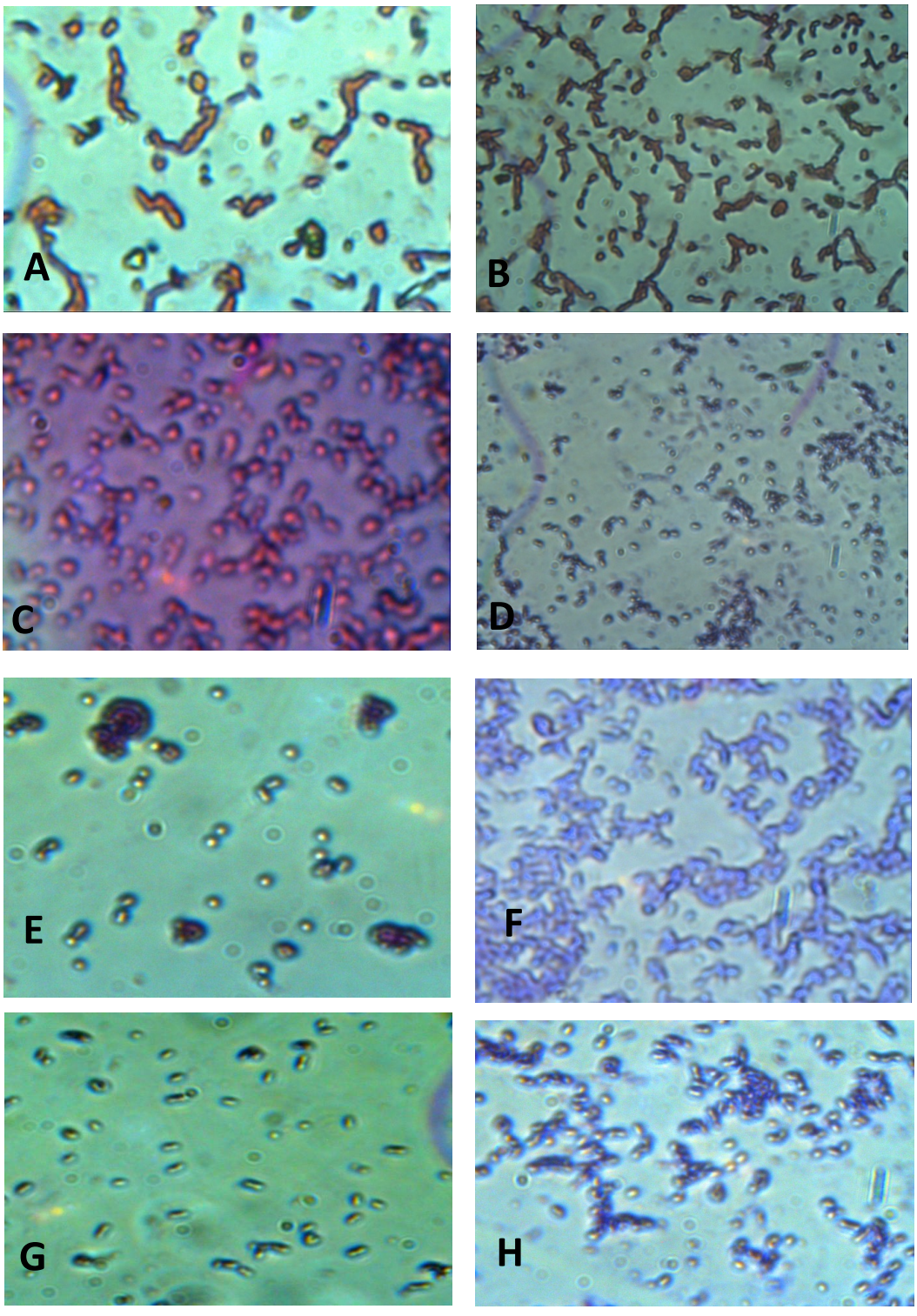


Plate 4.4 Photographs showing different bacteria after Gram staining

4.3.2 Biochemical test results of the selected isolates

Although morphological and cultural characteristics are essential for specific genus or species determination it also requires more information. The physiological or biochemical experiment will be helpful for the identification of bacterial strains. For identification, the following biochemical test was performed according to Bergy's Manual and finally compare to Micro-Rao online software to confirm identification.

Motility test results

For motility test the growth of the bacterial isolates were occurred in stab culture. Out of 44 isolates only 22 isolates (SGE-1, MSE-5, ISE-4, JGE-8, JSM-40, PLM-42, RGS-13, JSS-14, SGS-16, MLS-18, AGS-21, JSE-2, PLM-27, MSM-30, KSM-31, RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, ISM-44, RGE-35) showed positive results for motility test and rest of the isolates (ASS-15, MGS-17, RSS-23, MSS-24, PLS-19, RGS-20, IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, JSE-36,SGE-39, MGM-41, MSM-43, ISM-44, SSE-6, PLE-7, JGS-22, IGE-10, SGE-11, MGE-12) showed negative result. (Table 4.10 and Plate 4.5 A).

Indole test results

Indole test demonstrates the ability of certain bacteria to split the amino acid tryptophan to indole which accumulates in the medium. In this study 24 isolates (SGE-1, MSE-5, ISE-4, JGE-8, JSM-40, PLM-42, JSE-36, SGE-39, MGM-41, JSM-29, SSE-6, PLE-7, JGS-22, IGE-10, SGE-11, MGE-12, IGM-32, JGM-33, BSE-38, MLE-3, and MJE-9) showed positive results and produced red color. On the other hand, rest of the isolates (RGS-13, JSS-14, SGS-16, MLS-18, AGS-21, ASS-15, MGS-17, RSS-23, MSS-24, PLS-19, RGS-20, IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, IGM-32, JGM-33, BSE-38, MLE-3, MJE-9) showed negative results (yellow color) (Table 4.10 and Plate 4.5 D).

Oxidase test results

The oxidase test (also known as the cytochrome oxidase test) was used to look for oxidase enzymes produced by certain bacteria. Oxidases catalyze electron transport between substrates acting as electron donors in the bacterium and

tetramethyl-p-phenylenediamine or dimethyl-p-phenylenediamine- a redox dye present as the hydrochloride or oxalate salt (the latter has a longer shelf-life). In this study, all the isolated strain showed negative results for the oxidase test (Table 4.10 and Plate 4.6 B)

Catalase test results

Some of the bacteria exhibited a positive catalase test because it liberated gas (O_2) from hydrogen peroxide (H_2O_2) by enzymatic degradation. In this study, all the isolates showed positive results for the catalase test (Table 4.10 and Plate 4.5 B).

Citrate test results

In this study total 24 isolates (RGS-13, JSS-14, SGS-16, MLS-18, AGS-21, IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, JSE-2, PLM-27, MSM-30, KSM-31, JSE-36,SGE-39, MGM-41, JSM-29, SSE-6, PLE-7, JGS-22, IGE-10, SGE-11, MGE-12) showed positive result and produced blue color, and rest of the isolates showed negative results and produced yellow color (Table 4.10 and Plate 4.5 C).

Phenylalanine test results

For the phenylalanine test, only 13 isolates (IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35) showed positive result and produced green color but the rest of the isolates showed negative result (Table 4.10 and Plate 4.5 F).

Methyl red test results

This test was performed to identify mixed acid fomenter bacteria. In this case, 18 isolates (IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35) showed negative methyl red test by producing yellow or orange color indicating alkalinity and rest of the isolates showed positive result by the appearance of bright red color, indicating acidity (Table 4.10 and Plate 4.5 G).

Voges-Proskauer test

This test was done to detect the production of acetolin, due to the development of a red ring at the surface of the medium. In this study total 13 isolates (IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35) showed positive results for VP test because these isolates produced acetolin and 31 isolates showed negative results (Table 4.10 and Plate 4.6 A).

Kligler's iron agar (KIA) test

Enterobacteriaceae can ferment sucrose but not lactose. TSI reactions were based on the fermentation of lactose, dextrose, and glucose and the production of hydrogen sulfide. All the isolates showed yellow color in TSI butt, 19 isolates (RGS-13, JSS-14, SGS-16, MLS-18, AGS-21, ASS-15, MGS-17, RSS-23, MSS-24, PLS-19, RGS-20, RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35) showed red color in TSI slant and rest of the isolates showed yellow color in TSI slope. Moreover, among the 44 isolates only 23 isolates (RGS-13, JSS-14, SGS-16, MLS-18, AGS-21, JSE-36,SGE-39, MGM-41, JSM-29, SSE-6, PLE-7, JGS-22, IGE-10, SGE-11, MGE-12, RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35) showed positive in H_2S and produced black color and rest of the isolates were H_2S negative (Table 4.10 and Plate 4.6 C).

Glucose fermentation

For glucose fermentation test, only 22 isolates (RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35, IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, JSE-2, PLM-27, MSM-30, KSM-31) showed positive result and produced yellow color but rest of the isolates showed negative result (Table 4.10 and Plate 4.6 D)

Mannitol fermentation

For the mannitol fermentation test, only 8 isolates (RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, and RGE-35) showed negative result but the rest of the isolates showed positive results (Table 4.10 and Plate 4.6 E)

Sucrose fermentation

For **the** sucrose fermentation test, 30 isolates showed positive result and producing yellow color, and the rest of these 14 isolates (RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35, ASS-15, MGS-17, RSS-23, MSS-24, PLS-19, RGS-20) showed negative result (Table 4.10 and Plate 4.6 F).

Lactose Fermentation Test

For lactose fermentation test, only 33 isolates (ISE-4, JSE-2, IGM-32 and BSN-38 SGE-1, MSE-5, ISE-4, JGE-8, JSM-40, PLM-42, IGM-32, JGM-33, BSE-38, MLE-3, MJE-9, JSE-2, PLM-27, MSM-30, KSM-31, JSE-36,SGE-39, MGM-41, JSM-29, SSE-6, PLE-7, JGS-22, IGE-10, SGE-11, MGE-12, RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35) indicate positive results and 31 isolates showed negative results. Yellow color for acid production and gas as bubble in the Durham tube indicated positive results (Table 4.10 and Plate 4.6 G).

Table 4.10 Biochemical test results of the selected isolates

Isolates Code	Biochemical Test																	Suspected bacteria
	OX	IN	MO	CA	SH	CI	PH	MR	VP	KIA			Fermentation					
										Slope	Butt	H_2S	L	M	G	S		
SGE-1, MSE-5, ISE-4, JGE-8, JSM-40, PLM-42	-	+	+	+	+	-	-	+	-	Y	Y	-	+	+	+	+		*E. coli* sp.
JSS-14, MLS-18, AGS-21, RGS-13, SGS-16	-	-	+	+	+	+	-	+	-	R	Y	+	-	+	-	-		*Salmonella* sp.
ASS-15, MGS-17, RSS-23, MSS-24, PLS-19, RGS-20	-	-	-	+	+	-	-	+	-	R	Y	-	-	+	-	-		*Shigella* sp.
IGM-32, JGM-33, BSE-38, MLE-3, MJE-9	-	-	-	+	+	+	+	-	+	Y	Y	-	+	+	+	+		*Klebsiella* sp.
JSE-2, MSM-30, PLM-27, KSM-31	-	-	+	+	+	+	-	-	-	Y	Y	-	+	+	+	+		*Enterobacter* sp.
JSE-36,SGE-39, MGM-41, MSM-44, SSE-6, PLE-7, JGS-22, IGE-10, SGE-11, MGE-12	-	+	-	+	+	+	-	+	-	Y	Y	+	+	+	-	+		*Citrobacter* sp.
RGM-25, AGM-26, MLM-28, RSM-34, AGE-37,MSM-43 ISM-44,RGE-35	-	+	+	+	+	-	+	-	+	R	Y	+	+	-	+	+		*Proteus* sp.

[Key: OX = Oxidase, IN = Indole, MO = Motility, CA = Catalase, SH = Starch Hydrolysis, CI= Citrate, PH = Phenylalanine, MR = Methyl Red, VP = Voges-Proskauer, TSI = Triple Sugar Iron, H_2S = Hydrogen sulphide, LF = Lactose fermentation, SF = Sucrose fermentation, R = Red, Y = Yellow, + = Positive, - = Negative]

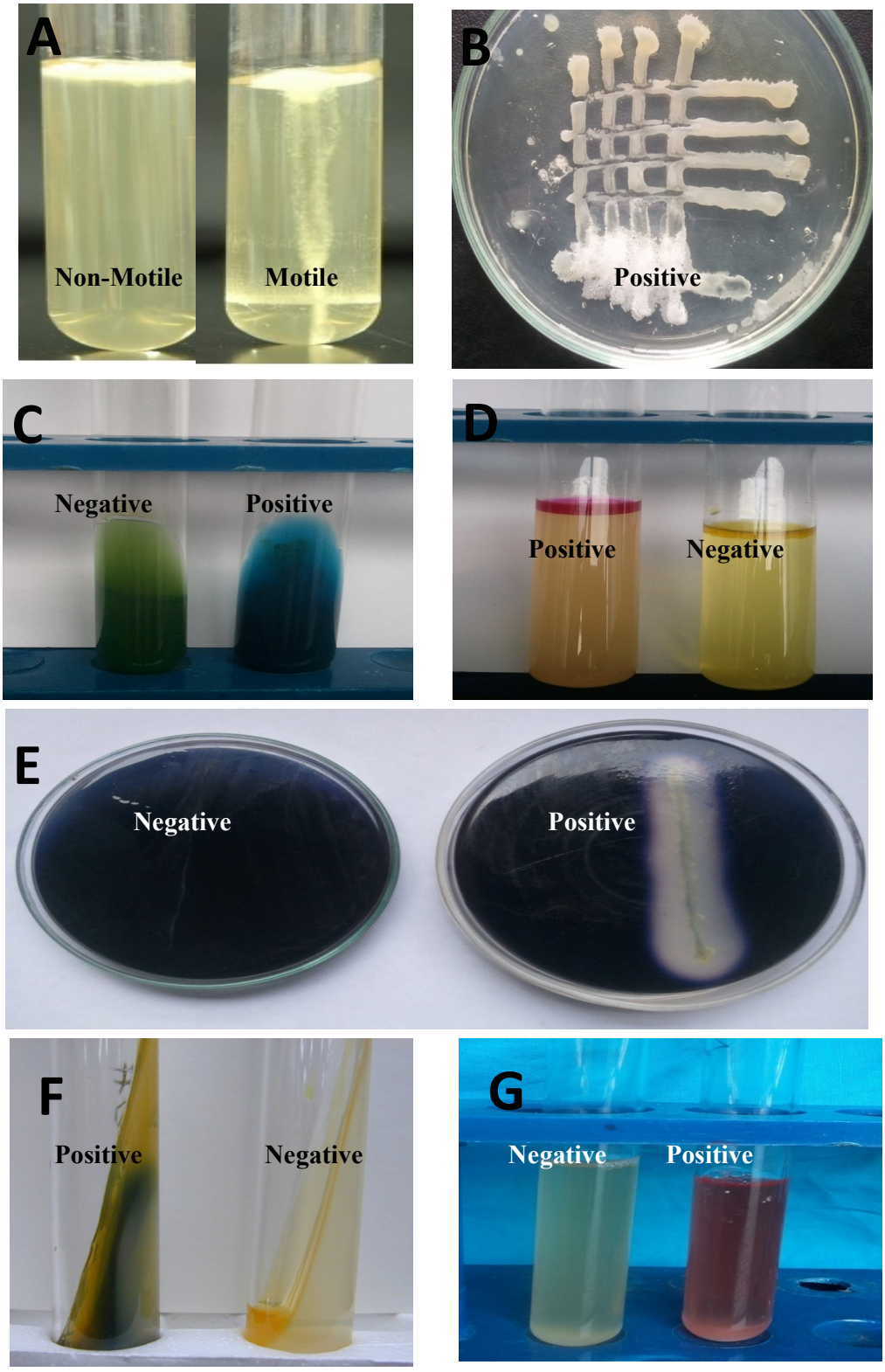


Plate 4.5 Photograph showing Motility test (A), Catalase test (B), Citrate test (C), Indole test (D), Starch hydrolysis test (E.), Phenylalanine test (F) and MR test (G)

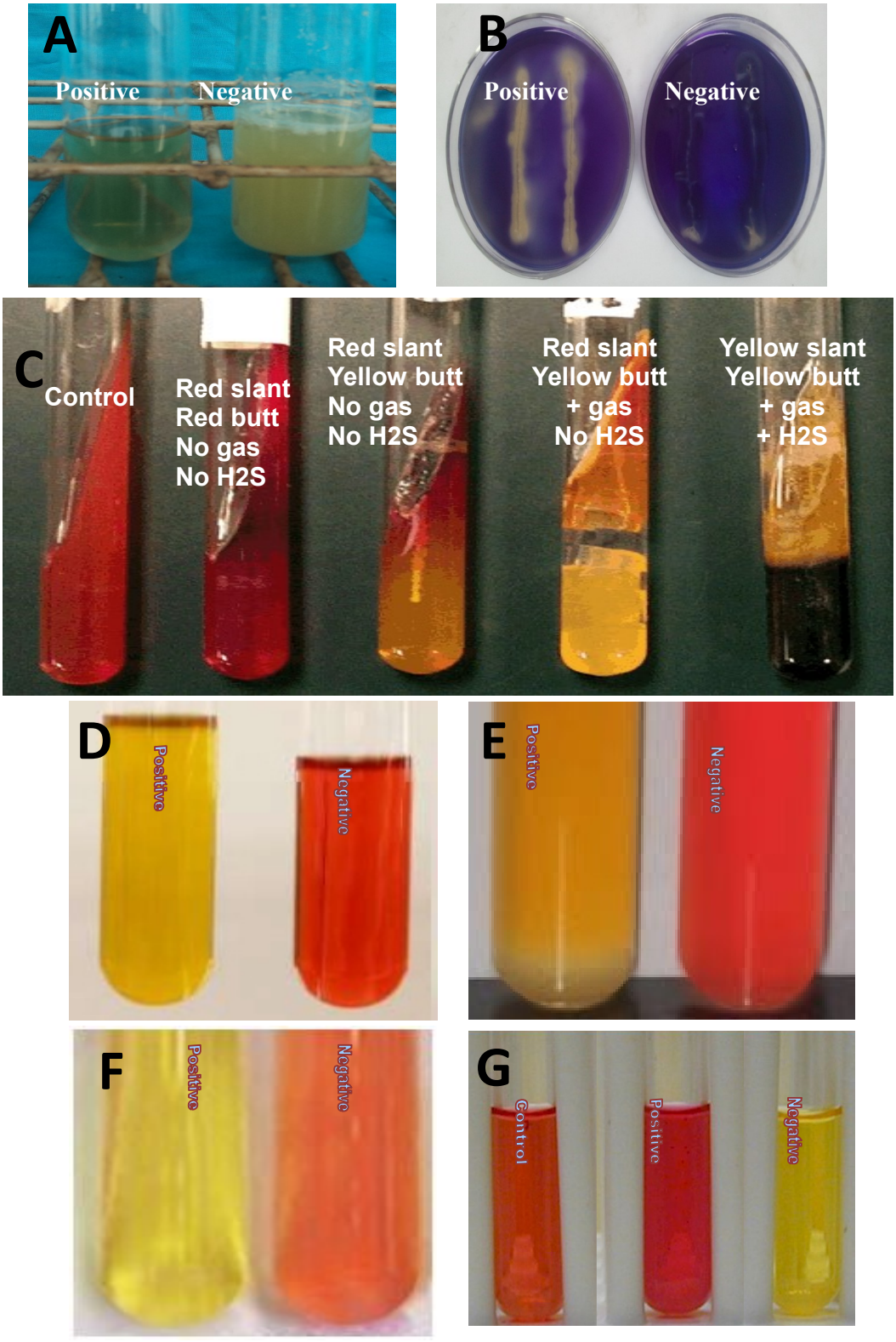


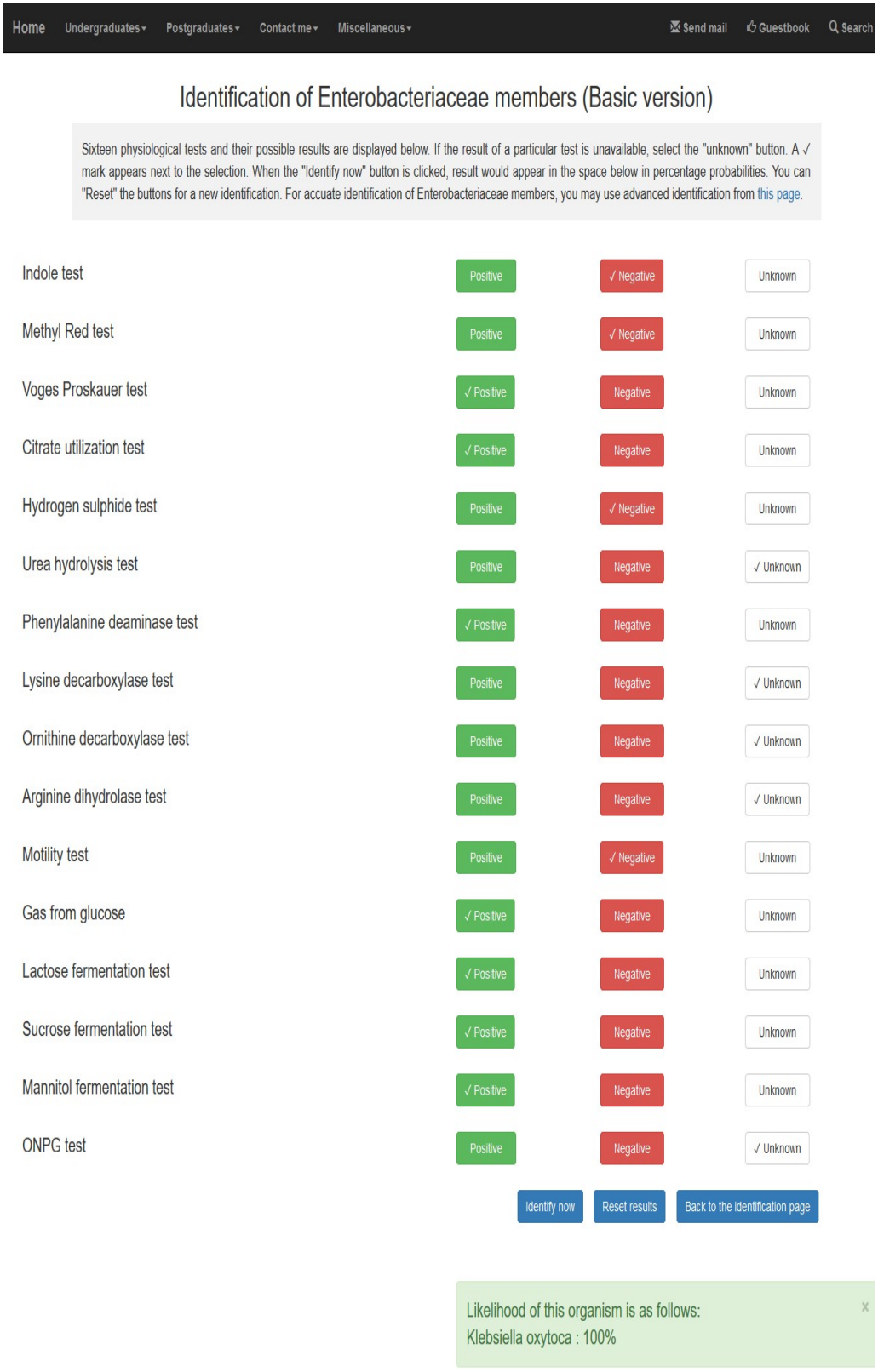
Plate 4.6 Photograph showing VP test (A), Oxidase test (B) and KIA test (C), Glucose fermentation test (D), Mannitol fermentation test (E), Sucrose fermentation test (F) and Lactose fermentation test (G)

4.3.3 Conformation of identification of the isolates with Micro-Rao online software

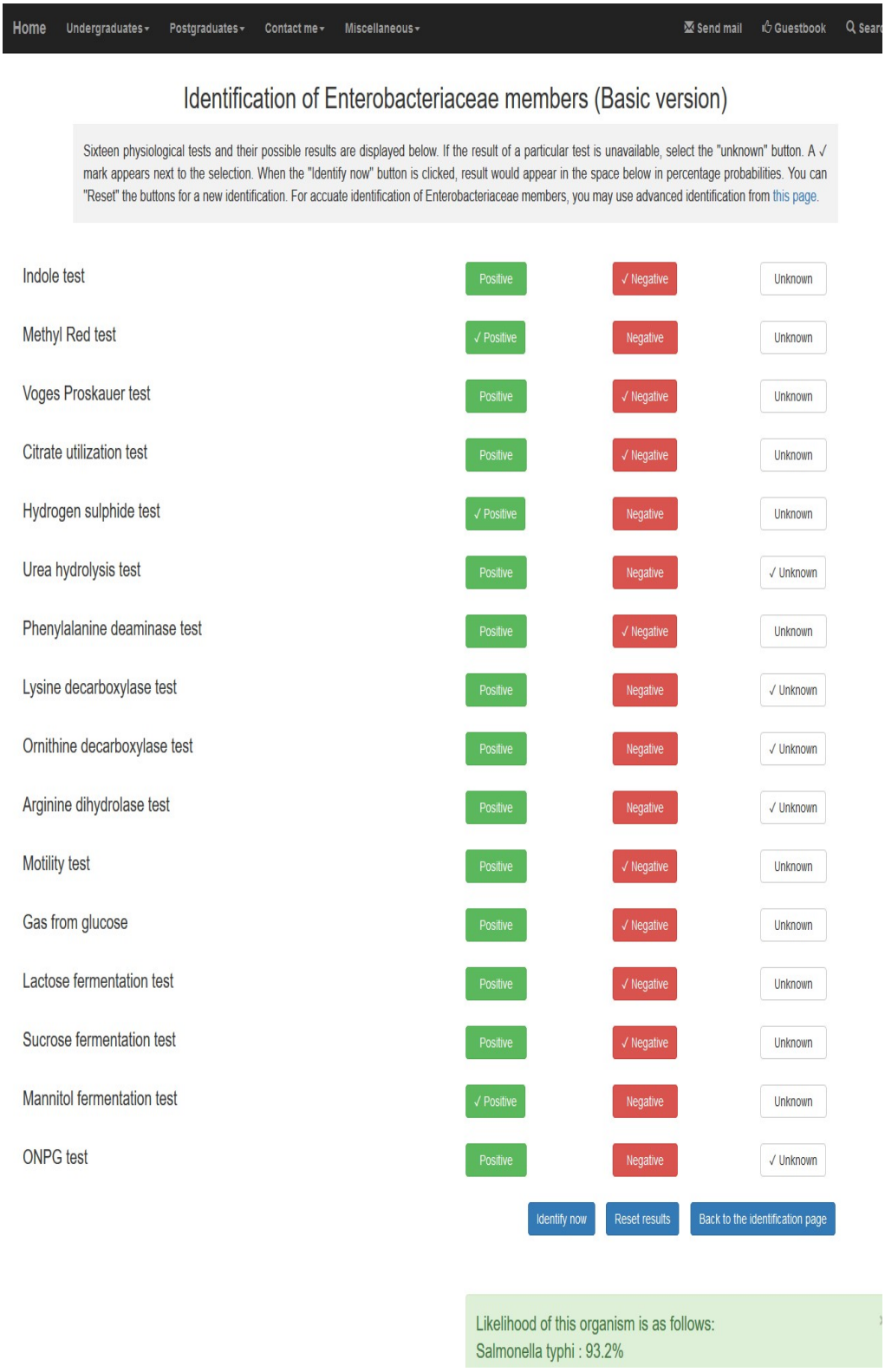
For the confirmation of the identification of the isolates, the results of biochemical tests had been inputted in Micro-Rao online software and the program showed the approximate name of bacterial species for the isolates with the percentage of probability. These results are presented as screen shot (Screenshot 1-7).

Screenshot 1. Result sheet for identification of the isolates (code no. SGE-1, MSE-5,ISE-4, JGE-8, JSM-40 and PLM-42)

Screenshot 2. Result sheet for identification of the isolates: (code no. MLE-3, MJE-9,MGS-17, IGM-32, JGM-33, BSE-38)

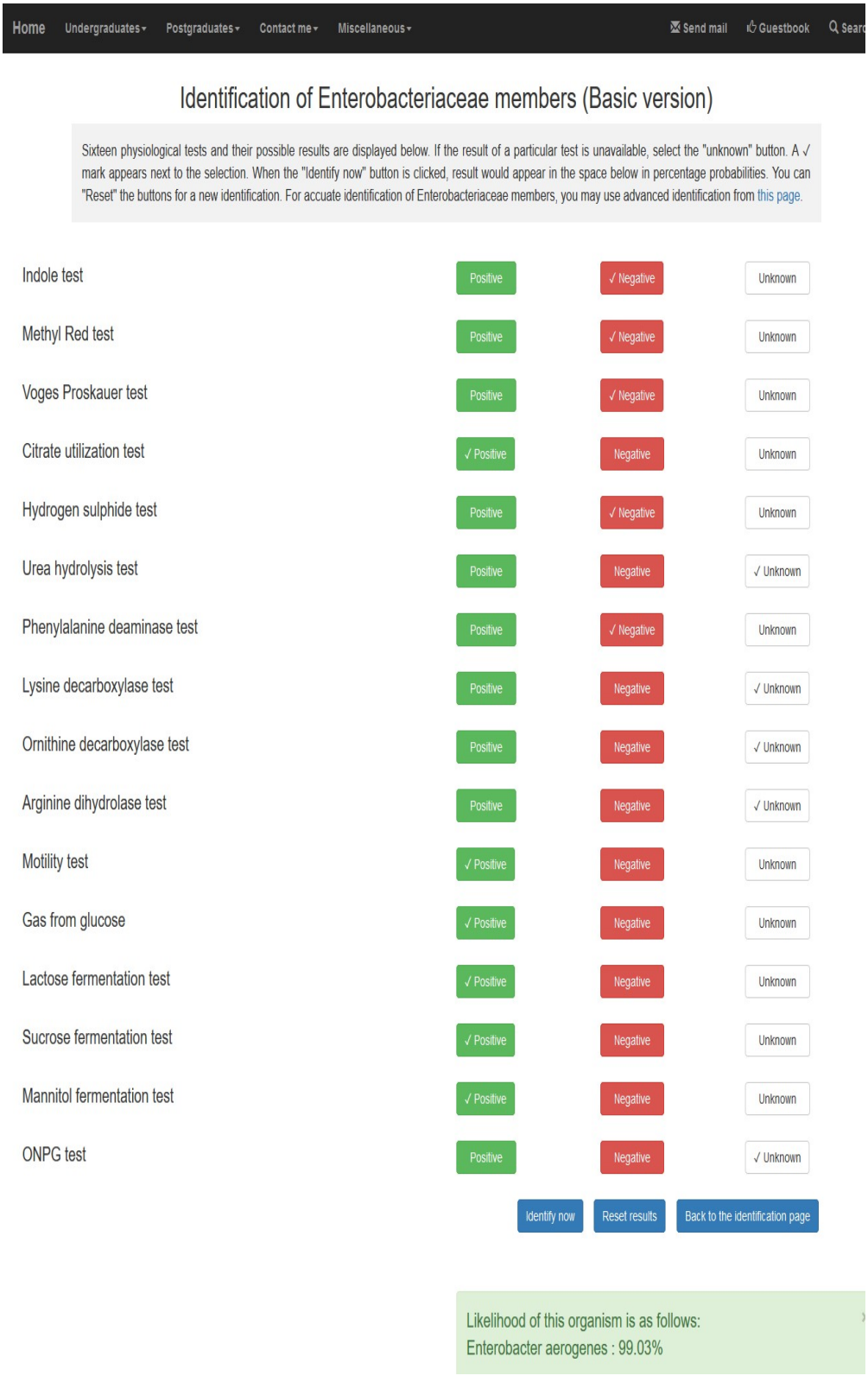


Screenshot 3. Result sheet for identification of the isolates: (code no. RGS-13, JSS-14, SGS-16, MLS-18, AGS-21)



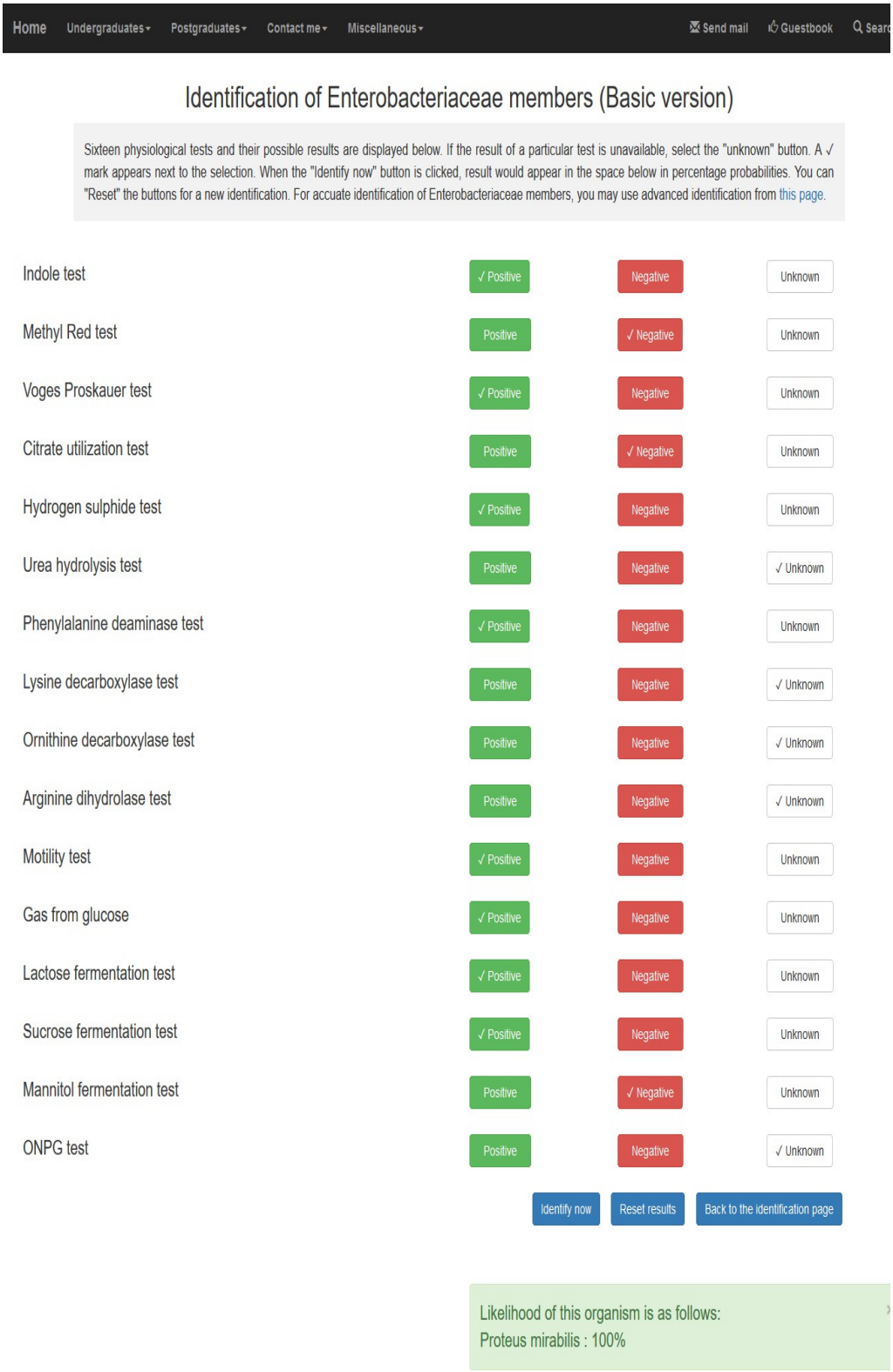
Screenshot 4. Result sheet for identification of the isolates: (code no. ASS-15, MGS-17, PLS-19, RGS-20, RSS-23, MSS-24)

Screenshot 5. Result sheet for identification of the isolates: (code no. JSE-2, MSM-30, PLM-27, KSM-31)



Screenshot 6. Result sheet for identification of the isolates: (code no. SSE-6, PLE-7, IGE-10, SGE-11, MGE-12, JGS-22, JSE-36, SGE-39, MGM-41, MSM-44)

Screenshot 7. Result sheet for identification of the isolates: (code no. RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35)



4.3.4 Confirmation of selected pathogenic bacteria

According to morphological and biochemical characteristics, the 44 isolated strains were identified which were conformed to Micro-Rao online software. The isolates SGE-1, MSE-5, ISE-4, JGE-8, JSM-40, PLM-42 were identified as *Escherichia coli* with the probability of 99.91%; Isolates MLE-3, MJE-9, IGM-32, JGM-33, BSE-38 were identified as *Klebsiella* sp. with the probability of 100% ; the isolates RGS-13, JSS-14, SGS-16, MLS-18, AGS-21 were identified as *Salmonella* sp. with the probability of 93.2%; the isolates ASS-15, MGS-17, PLS-19, RGS-20, RSS-23, MSS-24 were identified as *Shigella* sp. with the probability of 94.12%; the isolates JSE-2, MSM-30, PLM-27, KSM-31 were identified as *Enterobacter* sp. with the probability of 99.03%; the isolates SSE-6, PLE-7, IGE-10, SGE-11, MGE-12, JGS-22, JSE-36, SGE-39, MGM-41, MSM-44 were identified as *Citrobacter* sp. with the probability of 99.93% and the isolates RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35 were identified as *Proteus* sp. with the probability of 100%.

Finally, the identification of the isolates was confirmed as the seven species which are given below:

- *Escherichia coli* (isolate code SGE-1, MSE-5, ISE-4, JGE-8, JSM-40, PLM-42)
- *Klebsiella* sp. (isolate code MLE-3, MJE-9, IGM-32, JGM-33, BSE-38)
- *Salmonella* sp. (isolate code RGS-13, JSS-14, SGS-16, MLS-18, AGS-21)
- *Shigella* sp. (isolate code ASS-15, MGS-17, PLS-19, RGS-20, RSS-23, MSS-24)
- *Enterobacter* sp. (isolate JSE-2, MSM-30, PLM-27, KSM-31)
- *Citrobacter* sp. (SSE-6, PLE-7, IGE-10, SGE-11, MGE-12, JGS-22, JSE-36, SGE-39, MGM-41, MSM-44)
- *Proteus* sp. (RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35)

4.4 Pathogenicity test results of the isolated enterobacteria

The pathogenicity test of the isolates was carried out by hemolytic test, virulence test in poultry (through direct ingestion of inoculum), and Rose Bengal Agglutination Test (RBAT).

4.4.1 Hemolytic activity test results on blood agar

For the hemolytic test, the isolates were streaked on blood agar medium (Table 4.11 & Plate 4.7 A & B). Out of 44 isolates, twelve isolates (JSE-2, ISE-4, JGE-8, JSS-14, ASS-15, MGS-17, MLS-18, MSM-30, IGM-32, JGM-33, BSE-38 and JSM-40) showed positive β-hemolytic activity. These isolates were selected for further experiments.

Table 4.11 Hemolytic test results of the isolated enterobacteria in blood agar medium.

Sl No.	Isolates code	Hemolytic activity	Sl No.	Isolates code	Hemolytic activity
1	SGE-1	+	23	RSS-23	-
2	JSE-2	+++	24	MSS-24	-
3	MLE-3	+	25	RGM-25	+
4	ISE-4	+++	26	AGM-26	++
5	MSE-5	-	27	PLM-27	-
6	SSE-6	-	28	MLM-28	+
7	PLE-7	-	29	JSM-29	+
8	JGE-8	+++	30	MSM-30	+++
9	MJE-9	-	31	KSM-31	-
10	IGE-10	+	32	IGM-32	+++
11	SGE-11	-	33	JGM-33	+++
12	MGE-12	-	34	RSM-34	-
13	RGS-13	-	35	RGE-35	-
14	JSS-14	+++	36	JSE-36	-
15	ASS-15	+++	37	AGE-37	-
16	SGS-16	-	38	BSE-38	+++
17	MGS-17	+++	39	SGE-39	-
18	MLS-18	+++	40	JSM-40	+++
19	PLS-19	++	41	MGM-41	+
20	RGS-20	-	42	PLM-42	-
21	AGS-21	-	43	MSM-43	+
22	JGS-22	+	44	ISM-44	-

+++ = β hemolysis, ++ = α hemolysis, + = ɣ hemolysis, - = no hemolysis

Plate 4.7 Photographs showing pathogenicity test results: No hemolysis (A), β-hemolysis (B); direct ingestion of the hemolytic positive isolates in poultry (C), affected chicken (Dead) (D); RBAT test in a micro-titer plate (E).

4.4.2 Virulence test results of the isolates in poultry

Twelve β-hemolytic positive isolates were considered for the virulence test (Table 4.12). Out of 12 isolates, isolates JSS-20, MGS-17 and IGM-32 showed highly toxic reactivity and the chickens died after 6 to 9 days. Isolates JSE-2, ASS-15, MLS-18, JGM-35 and JSM-40 showed several sickness symptoms after 6 days while isolates JGE-8, MSM-30 and BSE-38 showed little sickness and isolate ISE-4 showed watery stool symptom. In control, no symptom was found and all the chickens were remained healthy.

Table 4.12 Virulence test results of the selected isolates in poultry

Sl. No.	Isolates code	Constant dose (1.5×10^8 cfu/ml)	Initial weight in (gm)	Weight loss after					Weight loss(%)	Symptoms (15days)
				3 days	6 days	9 days	12 days	15 days		
1	NC	-	672	672	672	672	672	672	Nill	Healthy
2	JSE-2	+	645	630	619	516	494	470	27.13	Sick
3	ISE-4	+	480	472	457	446	418	395	17.70	Watery stool
4	JGE-8	+	619	619	615	612	605	596	3.72	sick
5	JSS-14	+	635	619	---	---	---	---	-	Dead
6	ASS-15	+	479	476	472	463	460	458	4.38	Sick
7	MGS-17	+	645	632	522	---	---	---	-	Dead
8	MLS-18	+	658	655	648	641	636	632	3.95	Sick
9	MSM-30	+	587	585	578	571	565	558	4.94	Sick
10	IGM-32	+	475	461	---	---	---	---	-	Dead
11	JGM-33	+	625	622	615	611	605	601	3.84	Sick
12	BSE-38	+	555	543	515	490	410	390	29.72	Severe sickness
13	JSM-40	+	416	415	410	408	405	401	3.60	Sick

4.4.3 Rose Bengal Agglutination Test (RBAT) results of the isolates

For detection of the pathogenic potentiality of the selected isolates, the Rose Bengal Agglutination test (RBAT) was performed and the results are presented in Table 4.13 and (Plate. 4.7 E & Plate 4.8 D). From the table, it was observed that the reactivity (agglutination score) of sera in the RBAT varied from weak (++) to strong (++++). At the first fold, out of 12 only11 sera showed positive in RBAT: 2 reacted weakly (++), 3 reacted moderately (+++) and 6 reacted strongly (++++).

At the second fold, 9 sera showed positive in RBAT: 3 reacted weakly (++) and 6 reacted strongly (++++). In the case of 3rd and 4th fold-out of 12, only 6 sera reacted strongly (++++) and others showed negative results. This result also supports the clinical sign of poultry in an experimental study.

From the results, it was observed that the six bacteria namely as *Enterobacter* sp. (JSE-2), *E. coli* sp. (ISE-4), *Salmonella* sp. (JSS-14), *Shigella* sp. (MGS-17), *Klebsiella* sp. (IGM-32), *Klebsiella* sp. (BSE-38) showed strong agglutination reactivity in RBAT test which belongs in five species. Finally, the five pathogenic bacteria (*Enterobacter* sp., *E. coli* sp., *Salmonella* sp., *Shigella* sp., *Klebsiella* sp., and *Klebsiella* sp.) were selected for further experiment.

Table 4.13 Rose Bengal plate Agglutination Test (RBAT) result for the selected bacteria.

Bacteria (code)	1st fold	Scored (%)	2nd fold	Scored (%)	3rd fold	Scored (%)	4th fold	Scored (%)
Enterobacter sp.(JSE-2)	++++	100	++++	100	++++	100	++++	100
E. coli sp.(ISE-4)	++++	100	++++	100	++++	100	++++	100
E. coli sp.(JGE-8)	+ ++	75	++	50	+	25	-	0
Salmonella sp.(JSS-14)	++++	100	++++	100	++++	100	++++	100
*Shigella*_sp. (ASS-15)	+ +	50	+	25	-	0	-	0
Shigella sp. (MGS-17)	++++	100	++++	100	++++	100	++++	100
Salmonella sp. (MLS-18)	+	25	-	0	-	0	-	0
Enterobacter sp. (MSM-30)	++	50	+	25	-	0	-	0
Klebsiella sp.(IGM-32)	++++	100	++++	100	++++	100	++++	100
Klebsiella sp. (JGM-33)	+++	75	++	50	+	25	-	0
Klebsiella sp.(BSE-38)	++++	100	++++	100	++++	100	++++	100
E. coli sp. (JSM-40)	+++	75	+++	50	+	25	-	0

[Key: ++++=100% agglutination, +++=75% agglutination, ++=50% agglutination, +=25% agglutination while scored below 50 % (++) considered negative reactivity]

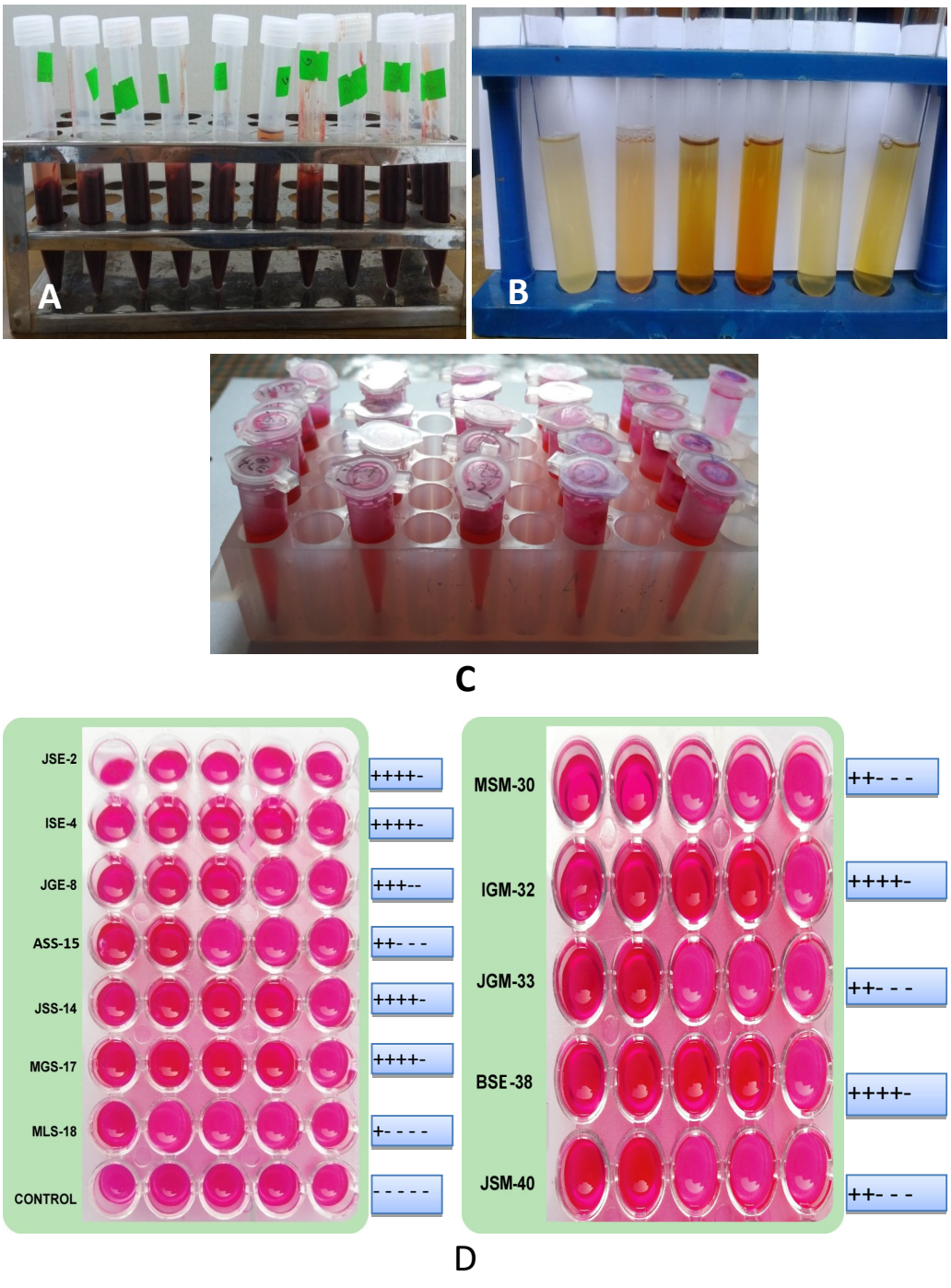


Plate 4.8 Photographs showing blood sample (immunized) (A); serum (B); antigen (C); RBAT test results in micro-titer plate (D).

4.5 *In vitro* screening of plant extracts against selected pathogenic bacteria for antibacterial properties

The present study was conducted to evaluate the antibacterial activity of the extracts of six plants i.e. *A. indica*, *C. papya, Z. officinale, C. dactylon, M. arvensis* and *P. pterocarpum* against the five pathogenic bacteria i.e. *Escherichia coli* (isolate code ISE-4), *Klebsiella* sp. (isolate code IGM-32 and BSE-38), *Enterobacter* sp. (isolate code JSE-2), *Salmonella* sp. (isolate code JSS-14) and *Shigella* sp. (isolate code MGS-17) using three solvent namely aqueous, ethanol and methanol.

4.5.1 Yield performance of the extract

The yield percentages of the plant extracts were varied (Table 4.14). The highest yield was obtained 15.5% in ethanol extract of *C. dactylon* while the lowest was 7.75% in *A. indica*. In the case of methanol extract, the highest yield was recorded 13.5% in *C. dactylon* and the lowest was 6.25% in *A. indica*. In case of aqueous extract, the highest yield was recorded 10.5% in *P. pterocarpum* and the lowest was 5.0% in *C. papaya.*

Table 4.14 Yield of six plant extracts

Name of Spices	Used parts	Powder (gm)	The solvent used (ml)			The yield of extracts (%)		
			Et	Met	Aq	Et	Met	Aq
C. dactylon	Leaves	40	200	200	300	15.5	13.5	10
Z. officinale	Rhizome	40	200	200	250	9.75	9.5	7.5
A. indica	Leaves	40	200	200	200	7.75	6.25	6.75
M. arvensis	Leaves	40	200	200	200	9.5	8.5	8.0
P. pterocarpum	Bark	40	200	200	200	14.25	12.0	10.5
C. papaya	Fruit	40	200	200	250	8.o	7.25	5.0

ET= Ethanol, MET= Methanol, AQ= Aqueous

Plate 4.9 Photographs showing different used plant parts and extracts.

4.5.2 Qualitative phytochemical analysis

Qualitative phytochemical analysis of the plant extracts was performed and the results are presented in Table 4.15. A cream-colored precipitate indicated the presence of respective alkaloid, brownish coloration indicated the presence of tannin and phenol, the formation of emulsion indicated the presence of saponin, greenish color in the lower chloroform layer indicated the presence of steroids, green color was indicated the presence of flavonoid and blue to the green color indicated the presence of glycoside (steroidal nucleus). Aqueous extracts of *A. indica, C. papaya and Z. officinale* showed positive results for alkaloid, tannin, saponin, steroid, and glycoside. *C. dactylon, M. arvensis* and *P. pterocarpum* showed negative result in case of alkaloids; tannins and alkaloids; tannins, flavonoids and steroids, respectively.

Table 4.15 Qualitative test results of plant extracts

Name	Phytoconstituents of plant extracts				
	Saponins	**Tannins**	**Alkaloids**	**Flavonoids**	**Steroids**
C.dactilon	+	+	-	+	+
Z. officinale	+	+	+	+	+
A.indica	+	+	+	+	+
M. arvensis	+	-	-	+	+
P. pterocarpum	+	-	+	-	-
C. papaya	+	+	+	+	+

+ = Present, - = Absent

4.5.3 Analysis of plant metabolites by HPLC (High-Performance Liquid Chromatography)

In the present investigation plant metabolites of selected plants were analyzed by HPLC method with Empower2™ software. Total 12 compounds (Oxalic acid, Ascorbic acid, Gallic acid, β-sitosterol, Glycine, Cysteine, Vanillic acid, Caffeic acid, Siringic acid, Phenylalanin, P-coumaric acid and Citrate) were used as standard phytochemicals. The retention time (RT) of those compounds was calculated in the laboratory by standard process and followed HPLC results reported by Zilani et al. (2016) as authentic standards.

The highest numbers of total compounds (30) were found in *A. indica* (Table 4.22). The next of compounds were detected as 27 for *C. papaya*, 19 for *P. pterocarpum*, 15 for *Z. officinale,* 14 for *M. arvensis* and the lowest numbers of compounds (12) were found in *C. dactylon.* The name of the compounds with their retention time (RT), total area and percentage of the area (%) and peak profiles of six used plant extracts are presented in Table 5.11-5.17 and Fig 5.1-5.6.

HPLC analysis of *A. indica* leaves extract

For *A. indica* leaves extract, total 30 numbers of phytochemicals were found (Table 4.16 and Fig 4.17). Comparing the peak profile with RT of standard compounds, total 12 numbers of phytochemicals were detected. The name of phytochemicals and their RT were as: Oxalic acid (3.888), Ascorbic acid (4.56), Galic acid (6.10), glycine (12.276), Cystein (12.73), Alanine (14.266), tryptophan (15.021), threonine (16.37), vanilic acid (20.612), Phenilalanine (24.614), Leusine (27.024), and citrate (30.402).

Table 4.16 HPLC analysis result of *A. indica* leaves extract

SL N.	Observed retention time (RT)	Suspected Phytocompound	Expected RT for suspected compound	Properties of detected compound		
				Area	% Area	Height
1	3.888	Oxalic acid	3.88	3946	0.16	354
2	4.699	Ascorbic acid	4.56	4954	0.2	524
3	5.849			53961	2.17	2989
4	6.19	Galic acid	6.10	41784	1.68	3139
5	7.204			23509	0.95	1225
6	10.383			27827	1.12	1696
7	10.651			45855	1.84	2373
8	10.986			38921	1.57	3081
9	11.241			71603	2.88	3541
10	12.276	Glycine	12.42	57969	2.33	4507
11	12.792	cystein	12.73	122040	4.91	7228
12	13.454			181433	7.3	12313
13	13.75			91161	3.67	5109
14	14.266	Alanine	14	321793	12.94	17982
15	15.021	Tryptophan	15	218109	8.77	8374
16	16.183			12842	0.52	1379
17	16.37	Threonine	16.6	114097	4.59	5959
18	17.152			267202	10.74	10702
19	17.983			37506	1.51	2700
20	20.612	Vanilic acid	20.09	43308	1.74	2129
21	23.899			7598	0.31	621
22	24.614	Phenylalanine	24.61	41686	1.68	2555
23	25.363			40128	1.61	2686
24	26.573			37441	1.51	2453
25	27.024	Leucine	17.45	20075	0.81	1404
26	29.155			4677	0.19	332
27	29.8			3398	0.14	291
28	30.402	Citrate	30.40	137370	5.52	7814
29	31.603			17642	0.71	1281
30	38.383			396930	15.96	1632

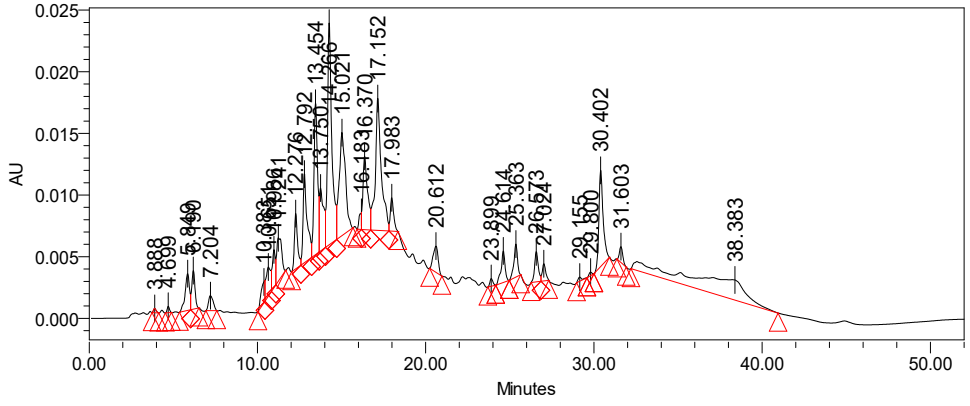


Fig 4.17 HPLC peak profile of *A. indica* leaf extract

HPLC analysis of *C. papaya* fruit extract

For *C. papaya* fruit extract total 27 numbers of phytochemicals were found and peak profile is presented in Table 4.17 and Fig 4.18. Comparing the peak profile with RT of standard compounds, total 10 numbers of phytochemicals were detected. The name of phytochemicals and their RT were as: ascorbic acid (4.559), β-sitosterol (7.493), glycine (12.239), cysteine (12.697), vanillic acid (19.970), caffeic acid (20.903), syringic acid (21.185), phenylalanine (24.61), p-coumaric acid (25.032) and citrate (28.02, 30.7)

Table 4.17 HPLC analysis result of *C. papaya* fruit extract

No.	Observed retention time (RT)	Suspected phytocompound	Expected RTfor suspected compound	Properties of detected compound		
				Area	% Area	Height
1	2.407			1977	0.07	187
2	2.639			12941	0.47	1539
3	4.366			43407	1.58	4705
4	4.559	Ascorbic acid	4.56	55568	2.02	4092
5	4.963			105486	3.84	5550
6	7.493	β-sitosterol	7.45	10574	0.39	811
7	11.974			137736	5.02	12439
8	12.239	Glycine	12.42, 13.2	533582	19.43	38860
9	12.697	Cysteine	12.73	94349	3.44	8667
10	12.844			211315	7.69	9611
11	13.632			61596	2.24	5294
12	14.078			105904	3.86	4861
13	14.967			1608	0.06	187
14	15.066			4285	0.16	560
15	15.492			35296	1.29	3023
16	15.779			22863	0.83	1787
17	16.350			4315	0.16	616
18	17.040			598935	21.81	39141
19	18.537			11881	0.43	1357
20	19.434			15416	0.56	1753
21	19.970	Vanillic acid	20.09	125385	4.57	7529
22	20.903	Caffeic acid	20.83	22020	0.8	1953
23	21.185	Syringic acid	21.18	24594	0.9	641
24	24.517			3971	0.14	581
25	24.583	Phenylalanine	24.61	7775	0.28	804
26	25.032	p-coumaric acid	25.1	459450	16.73	25127
27	29.571	Citrate	30.40	34030	1.24	1329

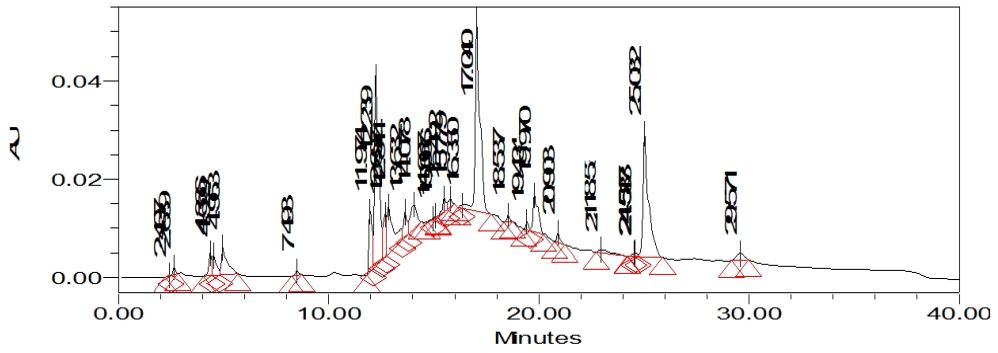


Fig 4.18 HPLC peak profile of *C. papaya* fruit extract

HPLC analysis of *Z. officinale* rhizome extract

For *Z. officinale* rhizome extract, total 15 numbers of phytochemicals were found and peak profile is presented in Table 4.18 and Fig 4.19. Comparing the peak profile with RT of standard compounds, total 8 numbers of phytochemicals were detected. The name of phytochemicals and their RT were as: Oxalic acid (2.47), Serine (10.867), tryptophan (14.914), Catechin (17.128), Phenilalanine (24.785), Leusine (27.097), Methinine (28.373), and citrate (30.49).

Table 4.18 HPLC analysis result of *Z. officinale* rhizome extract

No.	Observed retention time (RT)	Suspected Phytocompound	Expected RT for suspected compound	Properties of detected compound		
				Area	% Area	Height
1	2.47	Oxalic acid	2.7, 3.88	92786	7.17	5741
2	3.073			43964	3.4	3646
3	10.867	Serine	11	98868	7.64	1099
4	14.914	Tryptophan	15	24081	1.86	2016
5	17.128	Catechin	17.45	5411	0.42	484
6	17.979			8883	0.69	861
7	23.47			50222	3.88	2594
8	24.785	Phenylalanine	24.61	73025	5.64	4701
9	25.494			21515	1.66	1287
10	26.7			15954	1.23	1113
11	27.097	Leucine	27.3	6687	0.52	555
12	28.373	Methionine	28.3	90965	7.03	3788
13	29.267			225466	17.43	5125
14	29.967			211573	16.35	5775
15	30.49	Citrate	30.40	324496	25.08	11118

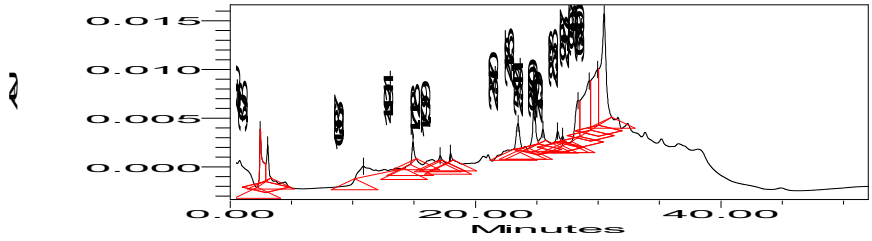


Fig 4.19 HPLC peak profile of *Z. officinale* rhizome extract

HPLC analysis of *M. arvensis* leaf extract

For *M. arvensis* leaf extract, total 14 numbers of phytochemicals were found and peak profile is presented in Table 4.19 and Fig 4.20. Comparing the peak profile with RT of standard compounds, total 7 numbers of phytochemicals were detected. The name of phytochemicals and their RT were as: Oxalic acid (2.874), Serine (11.154), tryptophan (15.241), threonine (16.32), Phenylalanine (24.638), Leusine (27.087) and citrate (30.31).

Table 4.19 HPLC analysis result of *M. arvensis* leaf extract

No.	Observed retention time (RT)	Suspected Phytocompound	Expected RT for suspected compound	Properties of detected compound		
				Area	% Area	Height
1	1.48			4406	0.19	498
2	2.525			775789	33.21	29990
3	2.874	Oxalic acid	2.7, 3.88	215363	9.22	15050
4	11.154	Serine	11.0	15602	0.67	1321
5	15.241	Tryptophan	15.0	25003	1.07	649
6	16.323	Threonine	16.6	157945	6.76	5256
7	18.034			18442	0.79	773
8	22.227			16733	0.72	1197
9	22.729			907973	38.86	43243
10	24.638	Phenylalanine	24.61	37730	1.61	2469
11	25.431			10568	0.45	858
12	26.681			15287	0.65	968
13	27.087	Leucine	27.3	7328	0.31	566
14	30.61	Citrate	30.40	128094	5.48	6843

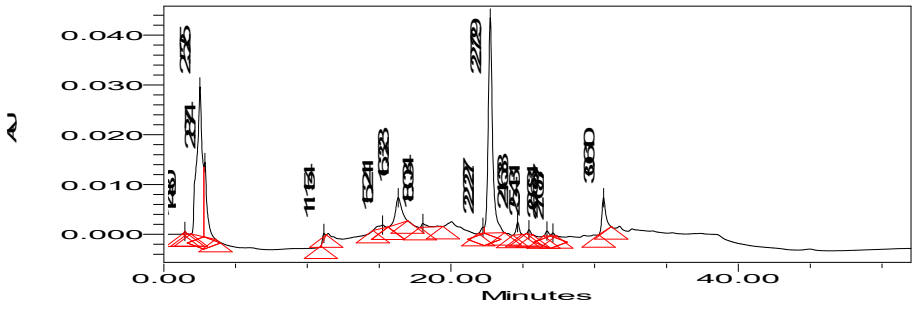


Fig 4.20 HPLC peak profile of *M. arvensis leaf* extract

HPLC analysis of *P. pterocarpum* bark extract

In *P. pterocarpum* bark extract, total 19 numbers of phytochemicals were found and peak profile is presented in Table 4.20 and Fig 4.21. Comparing the peak profile with RT of standard compounds, total 9 numbers of phytochemicals were detected. The name of phytochemicals and their RT were as: Oxalic acid (2.516), Serine (10.773), glycine (13.601), Alanine (14.273), tryptophan (14.943), threonine (17.05), Leusine (27.07), Methinine (28.171), and citrate (30.386).

Table 4.20 HPLC analysis result of *P. pterocarpum* bark extract

No.	Observed retention time (RT)	Suspected Phytocompound	Expected RT for suspected compound	Properties of detected compound		
				Area	% Area	Height
1	2.516	Oxalic acid	2.7, 3.7	19987	0.91	1146
2	3.07			22479	1.03	1920
3	10.773	Serine	11.0	117411	5.35	1467
4	13.601	Glycine	12.4, 13.20	95356	4.35	7299
5	14.273	Alanine	14.0	20380	0.93	1592
6	14.943	Tryptophan	15.0	25281	1.15	1067
7	17.05	Threonine	16.6	127150	5.8	1577
8	17.963			15851	0.72	1172
9	23.46			29972	1.37	1718
10	24.494			5995	0.27	503
11	25.411			35532	1.62	1998
12	26.209			7707	0.35	426
13	26.617			22375	1.02	1393
14	27.07	Leucine	27.3	7015	0.32	565
15	28.171	Methionine	28.3	8652	0.39	420
16	29.149			35271	1.61	985
17	30.386	Citrate	30.7	399481	18.22	11573
18	31.563			162488	7.41	3675
19	32.381			1034588	47.18	2767

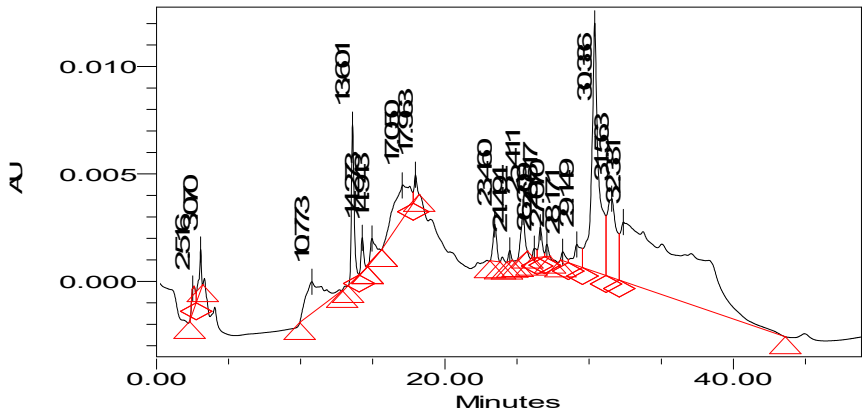


Fig 4.21 HPLC peak profile of *P. pterocarpum* bark extract

HPLC analysis of *C. dactylon* leaf extract

In *C. dactylon* leaf extract, total of 12 numbers of phytochemicals were found and peak profile is presented in Table 4.21 and Fig 4.22. Comparing the peak profile with RT of the standard compound, 7 numbers of phytochemicals were identified. The name of phytochemicals and their RT were as: Oxalic acid (2.874), Serine (11.154), tryptophan (15.241), threonine (16.32), Phenylalanine (24.638), Leusine (27.087) and citrate (30.31).

Table 4.21 HPLC analysis result of *C. dactylon* leaf extract

No.	Observed retention time (RT)	Suspected Phytocompound	Expected RT for suspected compound	Properties of detected compound		
				Area	% Area	Height
1	1.48			4406	0.19	498
2	2.525			775789	33.21	29990
3	2.874	Oxalic acid	2.7, 3.88	215363	9.22	15050
4	11.154	Serine	11.0	15602	0.67	1321
5	15.241	Tryptophan	15.0	25003	1.07	649
6	16.323	Threonine	16.6	157945	6.76	5256
7	18.034			18442	0.79	773
8	22.227			16733	0.72	1197
9	22.729			907973	38.86	43243
10	24.638	Phenylalanine	24.61	37730	1.61	2469
11	25.431			10568	0.45	858
12	26.681			15287	0.65	968
13	27.087	Leucine	27.3	7328	0.31	566
14	30.61	Citrate	30.40	128094	5.48	6843

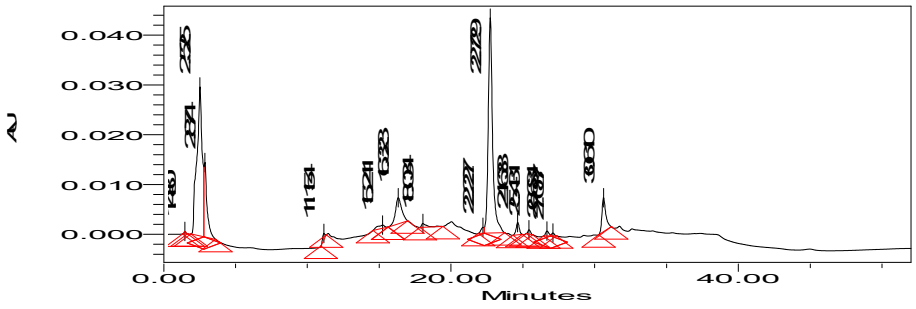


Fig 4.22 HPLC peak profile of *C. dactylon* leaf rhizome extract

Table 4.22 List of phytochemicals found (among used 12 standards) in studied plant extracts at a glance

Plant extract →		***A. indica***	***C. papaya***	***Z. officinale***	***M. arvensis***	***P. pterocarpum***	***C. dactylon***
Total phytocompound ditected by HPLC →		**30**	**27**	**15**	**14**	**19**	**12**
Standard phytochemicals ↓							
1	Oxalic acid	**D**	**-**	**D**	**D**	**D**	**D**
2	Ascorbic acid	**D**	**D**	**-**	**-**	**-**	**-**
3	Gallic acid	**D**	**-**	**-**	**-**	**-**	**-**
4	β-sitosterol	**-**	**D**	**-**	**-**	**-**	**-**
5	Glycine	**D**	**D**	**-**	**-**	**D**	**-**
6	Cysteine	**D**	**D**	**-**	**-**	**-**	**-**
7	Vanillic acid	**D**	**D**	**-**	**-**	**-**	**-**
8	Caffeic acid	**-**	**D**	**-**	**-**	**-**	**-**
9	Siringic acid	**-**	**D**	**-**	**-**	**-**	**-**
10	Phenylalanine	**D**	**D**	**D**	**D**	**-**	**D**
11	P-coumaric acid	**-**	**D**	**-**	**-**	**-**	**-**
12	Citrate	**D**	**D**	**D**	**D**	**D**	**D**

D = detected, - = not detected

4.5.4 Evalution of antibacterial activity of plant extracts against the pathogenic enterobacteria

In this study aqueous, methanol and ethanol extracts of *Azadirachta indica*, *Carica papya Zingiber officinale, Cynodon dactylon, Mentha arvensis* and *Peltophorum pterocarpum* were evaluated against five selected entero-pathogenic bacteria.

Antibacterial activity of *A. indica* leaf extract against the selected pathogens

Aqueous, methanol and ethanol extracts of *A. indica* at 150, 200, 250, 300 and 350 mg/ml concentrations were evaluated against five selected entero-pathogenic bacteria (Table 4.23). All the extracts showed the inhibitory effect against the pathogens without methanol and aqueous extract at 150 mg/ml concentration while no inhibition zone was observed. Ethanol extract showed a comparatively better result than methanol and aqueous extract.

The highest zone of inhibition was measured as 14.5 ± 0.28 mm at 350 mg/ml concentration against *Shigella* sp. for ethanolic leaf extracts. On the other hand, the lowest zone of inhibition was measured 10.8±0.44mm at the same concentration against *E. coli*.

For methanol extracts, the highest zone of inhibition was measured as 14.0 ± 0.57mm at 350 mg/ml concentration against *Shigella* sp while the lowest inhibition zone was 10±0.57mm at the same concentration against *E. coli*.

For aqueous leaf extracts, the highest zone of inhibition was measured as 11.5 ± 0.28mm at 350 mg/ml concentration against *Shigella* sp. while the lowest inhibition zone was 9.1±0.44mm at the same concentration against *Salmonella sp*

In all cases, the antibacterial activity of the extracts was compared with positive control and negative control. Positive control (disc containing 10µl tetracycline at 30 µg/ml concentration) exhibited zone of inhibition against all the pathogenic bacteria. But in the negative control (disc containing only distilled water) exhibited no zone of inhibition against all the pathogenic bacteria.

Table 4.23 Antibacterial activity of *A. indica* leaf extract against the pathogenic bacteria of poultry feed using disc diffusion method

Concentration (mg/ml)	Solvent	Zone of inhibition (mm)				
		***Salmonella* sp**	***Shigella sp***	***Escherichia coli***	***Enterobacter* sp**	***Klebsiella* sp**
150	ET	7.4±0.30	7.5±0.28	+	+	6.8±0.28
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
200	ET	7.5±0.28	8.8±0.44	+	8.8±0.44	7.6±0.33
	MET	7.1±0.44	7.5±0.28	+	8.7±0.28	7.1±0.28
	AQ	+	7.1±0.28	+	7.8±0.16	6.5±0.28
250	ET	8.5±0.28	10±0.57	10±0.28	8.1±0.16	8.5±0.44
	MET	7.6±0.44	9.33±0.33	7.3±0.44	8.3±0.57	7.8±0.44
	AQ	6.7±0.34	8.8±0.44	7.6±0.33	7.7±0.28	8.0±0.16
300	ET	10.8±0.44	12±0.57	9±0.57	10±0.57	10±0.57
	MET	9.5±0.28	9±0.28	9.3±0.33	9.6±0.16	9.7±0.57
	AQ	8.8±0.30	8.6±0.44	8.5±0.28	9.4±0.57	9.5±0.28
350	ET	12±0.57	14.5±0.28	10.8±0.44	11.5±0.28	13±0.57
	MET	11±0.57	14±0.57	10±0.57	10.5±0.28	11.7±0.60
	AQ	9.1±0.44	11.5±0.28	9.8±0.60	10±0.57	11.0±0.44
NC	ET	+	+	+	+	+
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
PC	CF	13.1±0.57	15±0.60	12.1±0.72	12.8±0.57	13.5±0.44

[Here, mean ± SEM of triplicate experiments, AQ= Aqueous, ET=Ethanol, MET= Methanol, NC= Negative control, PC= Positive control, CF= Ciprofloxacin antibiotic, "+" = Bacterial growth]

Antibacterial activity of *C. papaya* fruit extract against the selected pathogens

Aqueous, methanol and ethanol extracts of *C. papaya* at 150, 200, 250, 300 and 350 mg/ml concentrations were evaluated against five enteropathogenic bacteria and the results are presented in Table 4.24. All the extracts showed the inhibitory effect against the pathogens. Ethanol extract showed a comparatively better result than methanol and aqueous extract.

The highest zone of inhibition was measured as 14.8±0.44mm at 350 mg/ml concentration against *Shigella* sp. for ethanolic leaf extracts of *C. papaya.* On the other hand, the lowest zone of inhibition was measured 9.8±0.44mm at the same concentration against *Enterobacter sp*.

For methanol fruit extracts, the highest zone of inhibition was measured as 12.3± 0.88mm at 350 mg/ml concentration against *E. coli* sp. and the lowest inhibition zone as 9.5±0.28mm was measured at the same concentration against *Enterobacter sp*.

For aqueous fruit extracts, the highest zone of inhibition was measured as 10.8± 0.44mm at 350 mg/ml concentration against *Salmonella* sp. and the lowest inhibition zone as 8.4±0.41mm was measured at the same concentration against *Enterobacter sp*.

In all cases, the antimicrobial activity of the extracts was compared with positive control and negative control. Positive control (disc containing 10µl tetracycline at 30 µg/ml concentration) exhibited zone of inhibition against all the pathogenic bacteria. But negative control (disc containing only distilled water) exhibited no zone of inhibition against all the pathogenic bacteria.

Table 4.24 Antibacterial activity of papaya (*C. papaya*) fruit extracts against pathogenic bacteria of poultry feed using disc diffusion method.

Concentration (mg/ml)	Solvent	Zone of inhibition(mm)				
		***Salmonella* sp**	***Shigella* sp**	***Escherichia coli***	***Enterobacter* sp**	***Klebsiella* sp**
150	ET	6.5±0.28	7.4±0.30	+	+	+
	MET	+	+	+	6.5±0.15	+
	AQ	+	+	+	+	+
200	ET	7.9±0.37	9±0.57	7.5±0.26	7.5±0.15	8.2±0.57
	MET	7.3±0.44	8.5±0.44	+	+	7.8±0.29
	AQ	7.0±0.63	7.1±0.28	+	+	+
250	ET	10.4±0.29	10.5±0.28	9.8±0.66	8.5±0.44	9.6±0.57
	MET	8.6±0.30	9.8±0.44	6.5±0.29	8.1±0.16	8.4±0.60
	AQ	8.1±0.60	8.7±0.44	6.5±0.36	+	8.1±0.57
300	ET	12.5±0.23	12.8±0.60	11±0.57	9.3±0.57	10.8±0.28
	MET	10.5±0.28	11.6±0.88	9.0±0.44	8.4±0.28	9.8±0.44
	AQ	9.9±0.26	9.8±0.44	8.2±0.28	7.8±0.28	8.9±0.44
350	ET	14.1±0.44	14.8±0.44	13.6±0.88	9.8±0.44	13±0.57
	MET	12.1±0.44	12.1±0.60	12.3±0.88	9.5±0.28	11±0.28
	AQ	10.8±0.44	10.8±0.44	10±0.28	8.4±0.41	10.5±0.57
NC	ET	+	+	+	+	+
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
PC	CF	15±0.57	15.3±0.88	14±0.57	11.3±0.44	14.5±0.28

[Here, mean ± SEM of triplicate experiments, AQ= Aqueous, ET=Ethanol, MET= Methanol, NC= Negative control, PC= Positive control, CF= Ciprofloxacin antibiotic, "+" = Bacterial growth]

Antibacterial activity *Z. officinale* rhizome extract against the selected pathogens

Aqueous, methanol and aqueous extracts of *Z. officinale* rhizome at 150, 200, 250, 300 and 350 mg/ml concentrations were evaluated against five enteropathogenic bacteria and the results are presented in Table 4.25. All the extracts showed the inhibitory effect against the pathogens. Ethanol extract showed a comparatively better result than methanol and aqueous extract.

The highest zone of inhibition was measured as 14 ± 0.57 mm at 350 mg/ml concentration against *Shigella* sp. for ethanolic rhizome extract of *Z. officinale*. On the other hand, the lowest zone of inhibition was measured 11.8±0.44mm at the same concentration against *E. coli*.

For methanol rhizome extracts, the highest zone of inhibition was measured as 13.0 ± 0.57mm at 350 mg/ml concentration against *Klebsiella* sp. and the lowest inhibition zone as 10±0.57mm was measured at the same concentration against *Salmonella sp*.

For aqueous rhizome extracts, the highest zone of inhibition was measured as 11.8 ± 0.44mm at 350 mg/ml concentration against *Klebsiella* sp. and the lowest inhibition zone as 10±0.57mm was measured at the same concentration against *Enterobacter sp*

In all cases, the antimicrobial activity of the extracts was compared with positive control and negative control. Positive control (disc containing 10µl tetracycline at 30 µg/ml concentration) exhibited zone of inhibition against all the pathogenic bacteria. But negative control (disc containing only distilled water) exhibited no zone of inhibition against all the pathogenic bacteria.

Table 4.25 Antibacterial activity of ginger (*Z. officinale*) rhizome extracts against pathogenic bacteria of poultry feed using disc diffusion method

Concentration (mg/ml)	Solvent	Zone of inhibition (mm)				
		***Salmonella* sp**	***Shigella* sp**	***Escherichia coli***	***Enterobacter* sp**	***Klebsiella* sp**
150	ET	+	6.5±0.28	+	+	5±0.28
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
200	ET	6.1±0.44	7.8±0.44	+	6±0.57	7.5±0.28
	MET	6.5.5±0.28	6.8±0.44	+	6.5±0.28	6.5±0.28
	AQ	7±0.28	6.1±0.16	+	6.3±0.33	+
250	ET	8.0±0.28	9.9±0.57	7.1±0.44	8.5±0.28	9±0.57
	MET	7.7±0.16	9.2±0.28	6.8±0.4	7.1±0.16	7.8±0.44
	AQ	7.3±0.33	9±0.57	6.5±0.28	7±0.57	6.5±0.28
300	ET	9±0.57	11.8±0.44	9.8±0.57	10±0.57	10.5±0.28
	MET	8.5±0.28	10±0.57	9.1±0.16	9.7±0.57	10±0.57
	AQ	8.3±0.33	9.5.5±0.28	8.0±0.16	8.5±0.28	9±0.57
350	ET	12±0.57	14±0.57	11.8±0.44	12.8±0.44	14±0.57
	MET	10±0.44	12.8±0.44	10±0.57	11±0.57	13±0.55
	AQ	10.2±0.57	11±0.57	11±0.57	10±0.57	11.8±0.44
NC	ET	+	+	+	+	+
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
PC	CF	14±0.57	17.3±0.88	15.7±0.60	16±0.57	17.8±0.57

[Here, mean ± SEM of triplicate experiments, AQ= Aqueous, ET=Ethanol, MET= Methanol, NC= Negative control, PC= Positive control, CF= Ciprofloxacin antibiotic, "+" = Bacterial growth]

Antibacterial activity of *M. arvensis* leaves extract against the selected pathogens

Ethanol, methanol and aqueous extracts of *M. arvensis* leaves at 150, 200, 250, 300 and 350 mg/ml concentrations were evaluated against five enteropathogenic bacteria and the results are presented in Table 4.26. All the extracts showed the inhibitory effect against the pathogens. Ethanol extract showed a comparatively better result than methanol and aqueous extract.

The highest zone of inhibition was measured as 9.5 ± 0.12 mm at 350 mg/ml concentration against *E. coli*.for ethanol leaf extracts of *M. arvensis* . On the other hand, the lowest zone of inhibition was measured 8.2±0.55mm at the same concentration against *Enterobacter sp*.

For methanol leaf extracts, the highest zone of inhibition was measured as 14.0 ± 0.57mm at 350 mg/ml concentration against *Shigella* sp. and the lowest inhibition zone as 10±0.57mm was measured at the same concentration against *E. coli*.

For aqueous leaf extracts, the highest zone of inhibition was measured as 9.2 ± 0.67mm at 350 mg/ml concentration against *E. coli* .and the lowest inhibition zone as 8.0±0.45mm was measured at the same concentration against *Enterobacter sp*

In all cases, the antimicrobial activity of the extracts was compared with positive control and negative control. Positive control (disc containing 10µl tetracycline at 30 µg/ml concentration) exhibited zone of inhibition against all the pathogenic bacteria. But negative control (disc containing only distilled water) exhibited no zone of inhibition against all the pathogenic bacteria.

Table 4.26 Antibacterial activities of mint (*M. arvensis*) leaves extracts against enteropathogenic bacteria of poultry feed using disc diffusion method

Concentration (mg/ml)	Solvent	Zone of inhibition (mm)				
		***Salmonella* sp**	***Shigella* sp**	***Escherichia coli***	***Enterobacter* sp**	***Klebsiella* sp**
150	ET	+	+	+	+	+
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
200	ET	+	+	6.6±0.28	6.1±0.42	+
	MET	+	6.5±0.21	6.3±0.33	+	7.5±0.28
	AQ	+	6.3±0.33	+	7±0.22	+
250	ET	8.1±0.16	6.5±0.23	7±0.53	7.3±0.53	+
	MET	+	6.1±0.18	6.8±0.24	7.1±0.19	7.8±0.42
	AQ	+	6.1±0.43	6.1±0.17	7.0±0.23	7.5±0.28
300	ET	8±0.57	7.8±0.67	8.9±0.53	7.8±0.31	8.5±0.43
	MET	7.5±0.28	7.7±0.37	8.5±0.61	7.5±0.25	8.2±0.46
	AQ	7.3±0.33	8±0.27	8±0.37	7±0.53	8.1±0.45
350	ET	9±0.57	8.8±0.54	9.5±0.12	8.2±0.55	8.9±0.56
	MET	8.5±0.28	8.5±0.45	9.2±0.67	8±0.45	8.0±0.59
	AQ	8.2±0.57	8.2±0.51	8.9±0.27	8±0.53	7.8±0.44
NC	ET	+	+	+	+	+
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
PC	CF	14.5±0.28	17±0.51	18.2±0.42	15±0.51	15.8±0.55

[Here, mean ± SEM of triplicate experiments, AQ= Aqueous, ET=Ethanol, MET= Methanol, NC= Negative control, PC= Positive control, CF= Ciprofloxacin antibiotic, "+" = Bacterial growth]

Antibacterial activity of *C. dactylone leaves* extract against the selected pathogens

Ethanol, methanol and aqueous extracts of *C. dactylone leaves* at 150, 200, 250, 300 and 350 mg/ml concentrations were evaluated against five enteropathogenic bacteria and the results are presented in Table 4.27. All the extracts showed the inhibitory effect against the pathogens. Ethanol extract showed a comparatively better result than methanol and aqueous extract.

The highest zone of inhibition was measured as 9.0 ± 0.58mm at 350 mg/ml concentration against *Shigella* sp. for ethanol leaves extracts of *C. dactylone*. On the other hand, the lowest zone of inhibition was measured 7.9±0.56mm at the same concentration against *Klebsiella* sp

For methanol leaf extracts, the highest zone of inhibition was measured as 8.8± 0.54mm at 350 mg/ml concentration against *Shigella* sp. and the lowest inhibition zone as 7.8±0.59mm was measured at the same concentration against *Klebsiella sp*.

For aqueous leaf extracts, the highest zone of inhibition was measured as 8.6 ± 0.67mm at 350 mg/ml concentration against *Shigella* sp. and the lowest inhibition zone as 7.8±0.44mm was measured at the same concentration against *Klebsiella* sp.

In all cases, the antimicrobial activity of the extracts was compared with positive control and negative control. Positive control (disc containing 10µl tetracycline at 30 µg/ml concentration) exhibited zone of inhibition against all the pathogenic bacteria. But negative control (disc containing only distilled water) exhibited no zone of inhibition against all the pathogenic bacteria.

Table 4.27 Antibacterial activities of Durba (*C. dactylone*) leaves extracts against enteropathogenic bacteria of poultry feed using disc diffusion method

Concentration (mg/ml)	Solvent	Zone of inhibition (mm)				
		***Salmonella* sp**	***Shigella* sp**	***Escherichia coli***	***Enterobacter* sp**	***Klebsiella* sp**
150	ET	+	6.5±0.28	+	+	
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
200	ET	6.1±0.42		+		
	MET			+	6.5±0.21	6.5±0.28
	AQ	7±0.22	6.1±0.15	+	6.3±0.33	+
250	ET	7.3±0.53	7.9±0.51	7.1±0.41	6.5±0.23	
	MET	7.1±0.19	7.2±0.22	6.8±0.4	6.1±0.18	6.8±0.42
	AQ	7.0±0.23	7.5±0.59	6.5±0.21	6.1±0.43	6.5±0.28
300	ET	7.8±0.31	8.5±0.38	7.9±0.15	7.8±0.67	7.5±0.43
	MET	7.5±0.25	8.3±0.47	7.7±0.13	7.7±0.37	7.2±0.46
	AQ	7±0.53	8±0.40	7.8±0.58	8±0.27	7±0.45
350	ET	8.2±0.55	9±0.58	8.5±0.54	8.8±0.54	7.9±0.56
	MET	8±0.45	8.8±0.54	8±0.51	8.5±0.45	7.8±0.59
	AQ	8±0.53	8.6±0.67	8±0.44	8.2±0.51	7.8±0.44
NC	ET	+	+	+	+	+
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
PC	CF	15±0.51	16.3±0.80	15.7±0.60	17±0.51	16.8±0.55

[Here, mean ± SEM of triplicate experiments, AQ= Aqueous, ET=Ethanol, MET= Methanol, NC= Negative control, PC= Positive control, CF= Ciprofloxacin antibiotic, "+" = Bacterial growth]

Antibacterial activity of *P. pterocarpum* bark extract against the selected pathogens

Ethanol, methanol and aqueous extracts of *P. pterocarpum* bark at 150, 200, 250, 300 and 350 mg/ml concentrations were evaluated against five enteropathogenic bacteria and the results are presented in Table 4.28. All the extracts showed the inhibitory effect against the pathogens. Ethanol extract showed a comparatively better result than methanolic and aqueous extract.

The highest zone of inhibition was measured as 8.1 ± 0.61 mm at 350 mg/ml concentration against *Shigella* sp. for ethanol bark extracts of *P.pterocarpum*. On the other hand, the lowest zone of inhibition was measured 7.5±0.43mm at the same concentration against *Enterobacter*.

For methanol bark extracts, the highest zone of inhibition was measured as 7.8 ± 0.33mm at 350 mg/ml concentration against *Shigella* sp. and the lowest inhibition zone as 7.2±0.46mm was measured at the same concentration against *Enterobacter* sp.

For aqueous bark extracts, the highest zone of inhibition was measured as 7.5 ± 0.23mm at 350 mg/ml concentration against *Shigella* sp. and the lowest inhibition zone as 7±0.58mm was measured at the same concentration against *Klebsiella* sp.

In all cases, the antimicrobial activity of the extracts was compared with positive control and negative control. Positive control (disc containing 10µl tetracycline at 30 µg/ml concentration) exhibited zone of inhibition against all the pathogenic bacteria. But negative control (disc containing only distilled water) exhibited no zone of inhibition against all the pathogenic bacteria.

Table 4.28 Antibacterial activity of Radhachura (*P. pterocarpum*) bark extracts against entero-pathogenic bacteria of poultry feed using disc diffusion method

Concentration (mg/ml)	Solvent	Zone of inhibition (mm)				
		***Salmonella* sp**	***Shigella* sp**	***Escherichia coli***	***Enterobacter* sp**	***Klebsiella* sp**
150	ET	+	+	+	+	+
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
200	ET	+	+	+	+	+
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
250	ET	+	+	+	7.1±0.43	7.6±0.23
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
300	ET	7.3±0.16	7.1±0.23	7.5±0.53	7.7±0.53	+
	MET	+	7.0±0.17	7.4±0.24	7.1±0.17	7.8±0.42
	AQ	+	7.±0.42	7.1±0.17	7.0±0.22	7.5±0.28
350	ET	7.8±0.55	8.1±0.61	7.9±0.52	7.5±0.43	7.8±0.35
	MET	7.5±0.27	7.8±0.33	7.5±0.65	7.2±0.46	7.5±0.28
	AQ	7.1±0.39	7.5±0.23	7.4±0.36	7.1±0.45	7±0.58
NC	ET	+	+	+	+	+
	MET	+	+	+	+	+
	AQ	+	+	+	+	+
PC	CF	15.5±0.98	16.1±0.52	17.2±0.43	18±0.52	16.8±0.59

[Here, mean ± SEM of triplicate experiments, AQ= Aqueous, ET=Ethanol, MET= Methanol, NC= Negative control, PC= Positive control, CF= Ciprofloxacin antibiotic, "+" = Bacterial growth]

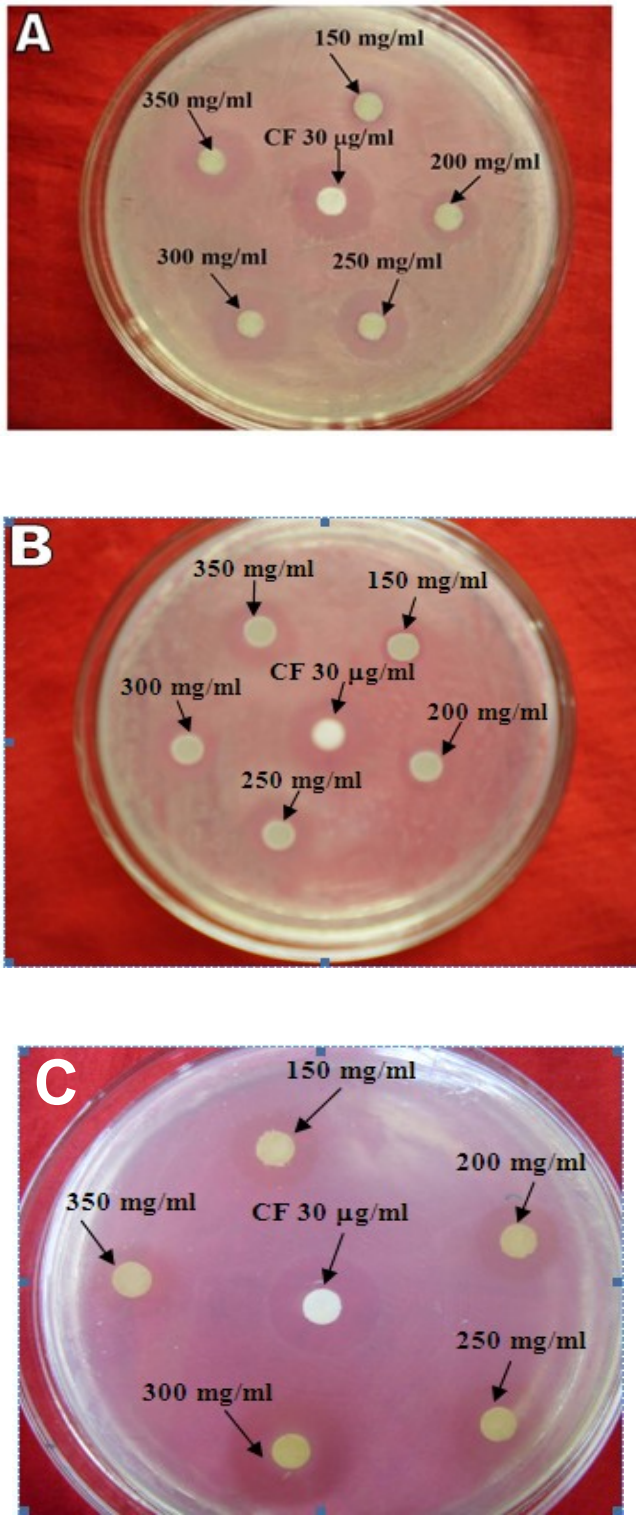


Plate 4.10 Photographs showing antibacterial activities of *C. papaya* plant extracts. ethanolic (A), methanolic (B) and aqueous (C).

Comparative analysis on zone of inhibition values among the six plants extracts, against the pathogenic bacteria

Comparative analysis on the zone of inhibition (mm) values of six plant extracts (in three solvents) against selected pathogenic bacteria are shown in Fig 4.23 and 4.24 using the summarized data of Table 4.29 to 4.30 at 350 mg/ml concentration. The results showed that among the six plant extracts, *C. papaya* was the best as creating a larger size of the zone of inhibition (14.8±0.44) against *Shigella* sp. The poorest performance occurred by *P. pterocarpum* which scored 7.0±0.58mm against *Klebsiella* sp. The rank of zone of inhibition of the plant extracts was found as *C. papaya* > *A. indica* > *Z. officinale* > *M. arvensis* > *C. dactylon* > *P pterocarpum.*

In a comparative analysis on the zone of inhibition (mm) values of six plants against selected pathogenic bacteria were investigated to find out the potentiality against five pathogenic bacteria to escape the lethal or antagonistic action of six plant extracts. From Fig 4.23 and 4.24, it was found that among the five enteropathogenic bacteria *Shigella* sp. showed the strongest microbial sensitivity. The rank of microbial sensitivity of the pathogenic bacteria was as *Shigella* sp. > *Salmonella* sp. >*E. coli* > *Klebsiella* sp. > Enterobacter sp.

Comparative analysis on the zone of inhibition (mm) values of three solvents against the pathogenic bacteria was done to detect which solvent played a better role. From the comparison, it reveals that among the three solvents ethanol played the best role and the rank was as Ethanol > Methanol > Aqueous.

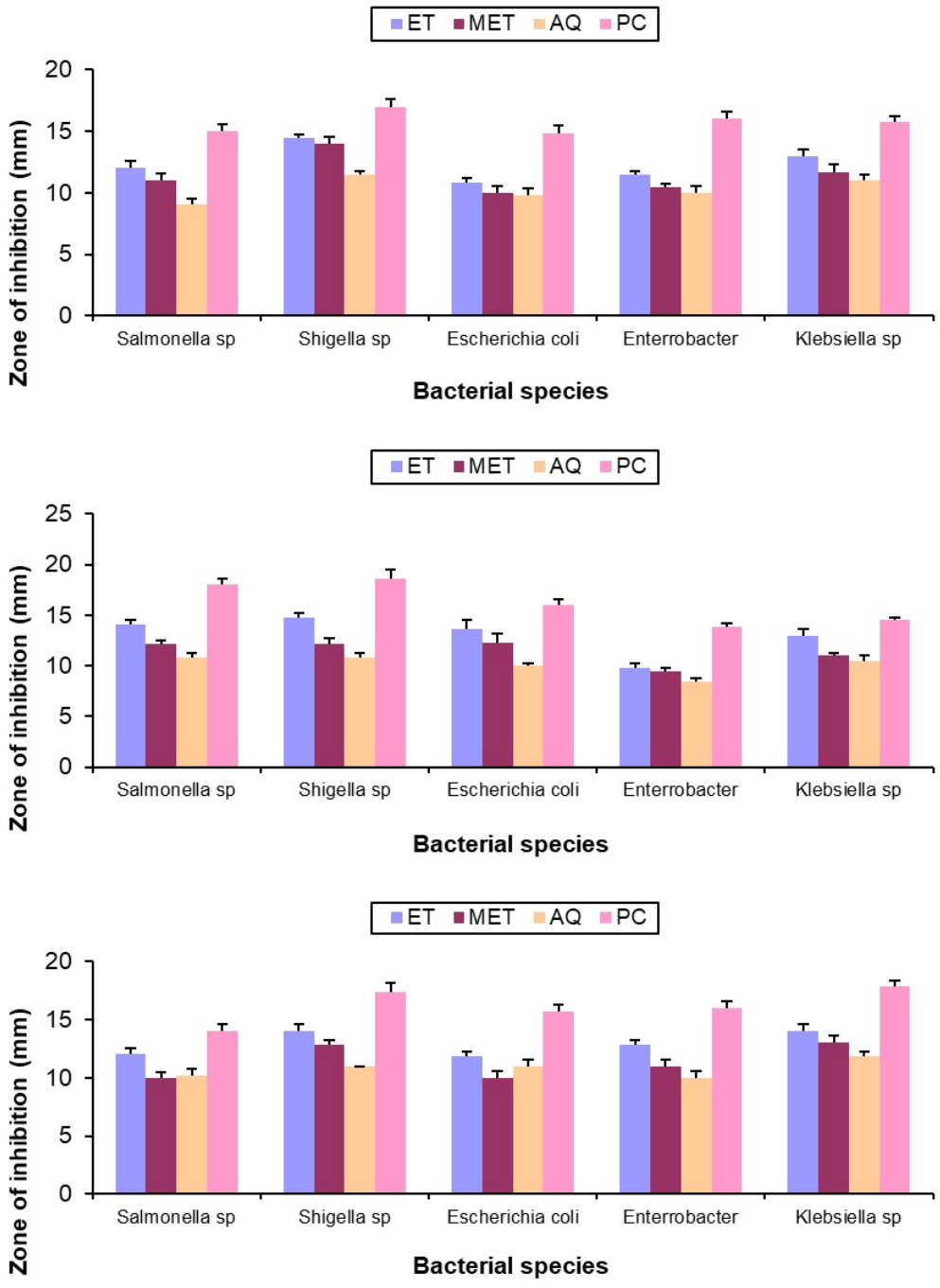


Fig 4.23 Comparison of zone of inhibition of *A. indica* leaf, *C. papaya* fruit & *Z. officinale* rhizome extracts (350mg/ml) against pathogenic bacteria.

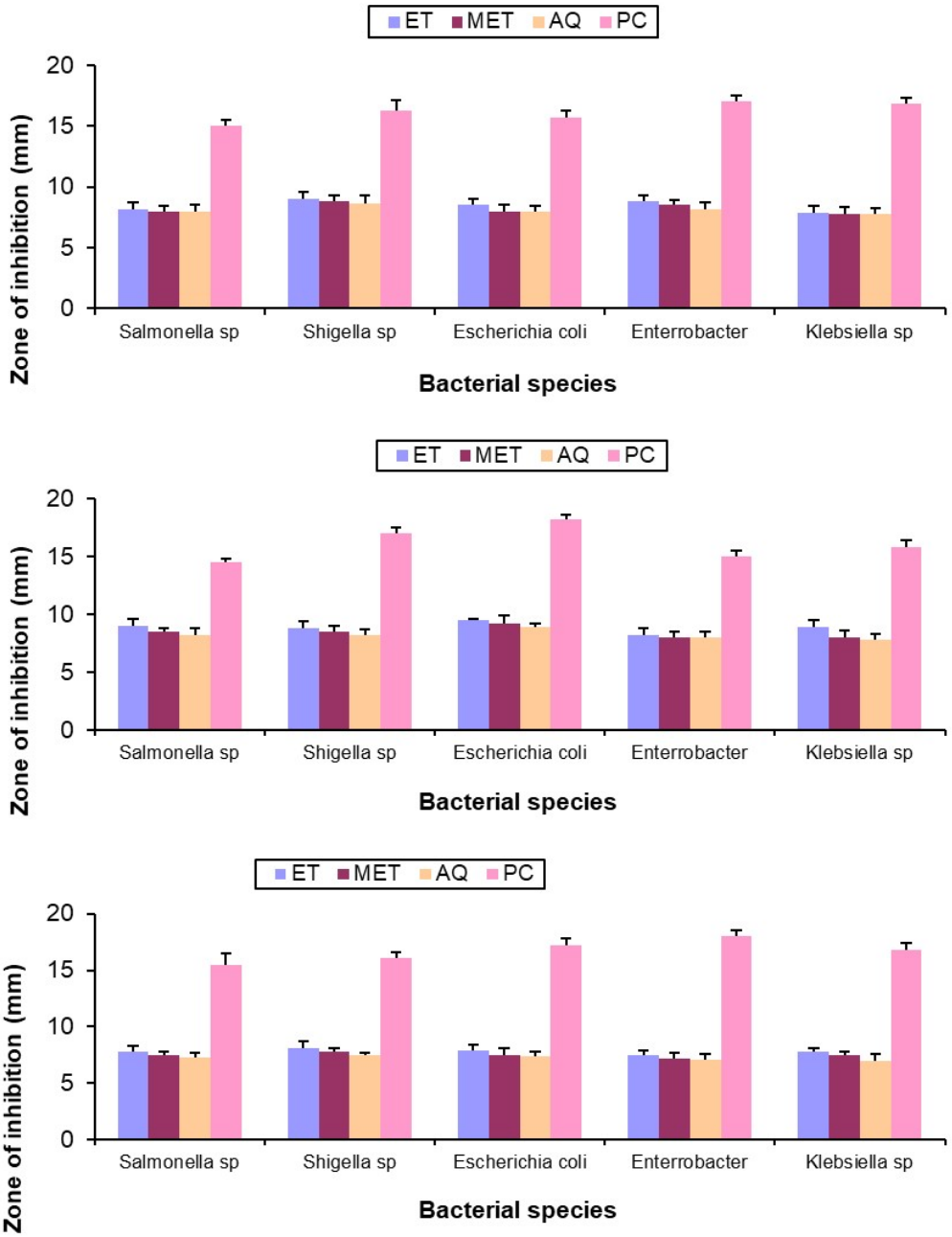


Fig 4.24 Comparison of zone of inhibition of *C. dactylon*, *M. arvensis* and *P. pterocarpum* extracts (350mg/ml) against pathogenic bacteria.

4.5.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of six plant extracts against selected pathogenic bacteria

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium and the MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculums by ≥99.9%. The results of MIC and MBC of six plant extracts against five pathogenic bacteria are shown in Table 4.29 and 4.30. The results reveals that the selected plant extracts showed a varying degree of MIC and MBC.

MIC and MBC for *A. indica*

The results of MIC and MBC screening of aqueous, methanol, and ethanol extracts of the *A. indica* are shown in Table 4.29 and 4.30. The results reveal that the MIC values were ranging from 50 to 150 mg/ml and MBC were ranged from 75-175 mg/ml against the pathogenic bacteria. For ethanolic extract, the ranges of MIC value were calculated as 50-125 mg/ml and MBC were 75-150 mg/ml. In the case of methanol, the values of MIC were ranged from 50-125 mg/ml and MBC were ranged 75-150 mg/ml while in aqueous it found to be 75-150 mg/ml and 100-175 mg/ml, respectively.

MIC and MBC for *C. papaya*

The results of MIC and MBC screening of aqueous, methanol and ethanol extracts of *the C. papaya* are shown in Table 4.29 and 4.30. The results reveal that the MIC values were ranging from 50 to 150 mg/ml and MBC were ranged from 75-175 mg/ml. For ethanolic extract, the ranges of MIC value were calculated as 50-125 mg/ml and MBC was 100-150 mg/ml. In case of methanol, the values of MIC were ranged from 50-125 mg/ml and MBC were ranged from 75-150 mg/ml, in aqueous it found to be 100-150 mg/ml and 125-150 mg/ml, respectively.

MIC and MBC for *Z. officinale*

The results of MIC and MBC screening of aqueous, methanol and ethanol extracts of the *Z. officinale* are shown in Table 4.29 and 4.30. The results reveal that the MIC values were ranging from 50 to 150 mg/ml and MBC were ranged from 75-175 mg/ml. For ethanolic extract, the ranges of MIC value were calculated as 50-100 mg/ml and MBC was 75-125 mg/ml. In the case of methanol, the values of MIC were ranged from 100-150 mg/ml and MBC were ranged from 125-150 mg/ml, in aqueous it found to be 50-150 mg/ml and 125-175 mg/ml, respectively.

MIC and MBC for *M. arvensis*

The results of MIC and MBC screening of aqueous, methanol, and ethanol extracts of the *M. arvensis* are shown in Table 4.29 and 4.30. The results reveal that the MIC values were ranging from 50 to 125 mg/ml and MBC were ranged from 75-150 mg/ml. For ethanolic extract, the ranges of MIC value were calculated as 50-100 mg/ml and MBC was 75-125 mg/ml. In case of methanol, the values of MIC were ranged from 75-150 mg/ml and MBC were ranged from 100-150 mg/ml, in aqueous it found to be 125-150 mg/ml and 125-175 mg/ml, respectively.

MIC and MBC for *C. dactylone*

The results of MIC and MBC screening of aqueous, methanol, and ethanol extracts of the *C. dactylone* are shown in Table 4.29 and 4.30. The results reveal that the MIC values were ranging from 75 to 150 mg/ml and MBC were ranged from 100-175 mg/ml. For ethanolic extract, the ranges of MIC value were calculated as 75-125 mg/ml and MBC was 100-150 mg/ml. In the case of methanol, the values of MIC were ranged from 100-125 mg/ml and MBC were ranged 125-150 mg/ml, in aqueous it found to be 125-150 mg/ml and 150-175 mg/ml, respectively.

MIC and MBC for *P. pterocarpum*

The results of MIC and MBC screening of aqueous, methanol, and ethanol extracts of the *P. pterocarpum* are shown in Table 4.29 and 4.30. The results reveal that the MIC values were ranging from 50 to 150 mg/ml and MBC were ranged from 75-175 mg/ml. For ethanolic extract, the ranges of MIC value were calculated as

50-125 mg/ml and MBC was 75-150 mg/ml. In case of methanol, the values of MIC were ranged from 75-150 mg/ml and MBC were ranged 125-175 mg/ml, in aqueous it found to be 100-150 mg/ml and 150-175 mg/ml, respectively.

Table 4.29 Minimum inhibitory concentration (MIC) of six plant extracts against selected pathogenic bacteria

Plant used	Bacteria	MIC value ($mgml^{-1}$)		
		Ethanol	Methanol	Aqueous
A. indica	*Salmonella* sp	50	50	75
	Shigella sp	75	50	100
	Escherichia coli	100	125	125
	Enterobacter sp	125	125	150
	Klebsiella sp	100	125	125
C. papaya	*Salmonella* sp	75	50	100
	Shigella sp	100	125	125
	Escherichia coli	125	125	150
	Enterobacter sp	100	125	100
	Klebsiella sp	50	75	125
Z. officinale	*Salmonella* sp	50	100	50
	Shigella sp	75	100	125
	Escherichia coli	100	150	125
	Enterobacter sp	100	125	150
	Klebsiella sp	75	125	150
M. arvensis	*Salmonella* sp	75	75	150
	Shigella sp	100	100	125
	Escherichia coli	50	100	150
	Enterobacter sp	100	150	125
	Klebsiella sp	75	100	125
C. dactylon	*Salmonella* sp	100	100	125
	Shigella sp	125	125	150
	Escherichia coli	75	100	125
	Enterobacter sp	100	125	125
	Klebsiella sp	75	100	125
P. pterocarpumm	*Salmonella* sp	50	75	150
	Shigella sp	75	100	100
	Escherichia coli	100	125	150
	Enterobacter sp	100	125	125
	Klebsiella sp	125	150	150

Table 4.30 Minimum bactericidal concentration (MBC) of six plant extracts against selected pathogenic bacteria

Plant used	Bacteria	MBC value ($mgml^{-1}$)		
		Ethanol	Methanol	Aqueous
A. indica	*Salmonella* sp	75	75	100
	Shigella sp	100	75	125
	Escherichia coli	125	150	150
	Enterobacter sp	150	150	175
	Klebsiella sp	125	150	150
C. papaya	*Salmonella* sp	100	75	125
	Shigella sp	125	150	150
	Escherichia coli	150	150	150
	Enterobacter sp	125	150	150
	Klebsiella sp	100	125	150
Z. officinale	*Salmonella* sp	75	125	125
	Shigella sp	125	125	150
	Escherichia coli	125	150	175
	Enterobacter sp	125	150	175
	Klebsiella sp	125	125	125
M. arvensis	*Salmonella* sp	125	100	125
	Shigella sp	125	150	150
	Escherichia coli	75	125	150
	Enterobacter sp	125	150	175
	Klebsiella sp	100	125	175
C. dactylon	*Salmonella* sp	125	150	150
	Shigella sp	150	150	175
	Escherichia coli	100	125	150
	Enterobacter sp	125	150	175
	Klebsiella sp	100	125	150
P. pterocarpumm	*Salmonella* sp	75	125	150
	Shigella sp	100	150	150
	Escherichia coli	150	150	175
	Enterobacter sp	125	150	150
	Klebsiella sp	150	175	175

Comparative analysis MIC and MBC of six plants extracts against selected pathogenic bacteria

Comparative analysis was done to find out the best plant extract for controlling selected pathogenic bacteria. Average MIC and MBC values (obtained from Table 4.29 and 4.30) of six plant extracts against selected pathogenic bacteria were used for this and the results were shown in Figure 4.25 and 4.27. From the results, it reveals that *A. indica* performed best for inhibiting and the poorest performance was shown *P. pterocarpum*. Considering the average MIC values, the rank of potentiality of six plant extracts were as *A. indica* > *C. papaya* > *Z. officinale* > *M. arvensis* > *C. dactylon* > *P. pterocarpum*

For MBC *A. indica* showed the best result for killing bacteria in minimum concentration and the poorest *P. pterocarpum*. Considering the average MBC values, the rank of potentiality of six plants was *A. indica* > *C. papaya* > *Z. officinale* > *M. arvensis* > *C. dactylon* > *P. pterocarpum*

Comparative analysis on microbial susceptibility of selected pathogenic bacteria to six plant extracts was done. The resultreveals that *Salmonella*, *E. coli* sp. was the most susceptible strain against plant extract as it showed 116.67 and 148.61mg/ml MIC and MBC values, respectively. The rank of microbial susceptibility to plant extracts was as *E. coli*. > *Enterobacter* sp. > *Klebsiella* sp. > *Shigella* sp. > *Salmonella* sp.

In comparative analysis, it is revealed that the ethanol was the best solvent and the rank of solvents according to their performance was as Ethanol > Methanol > Aqueous.

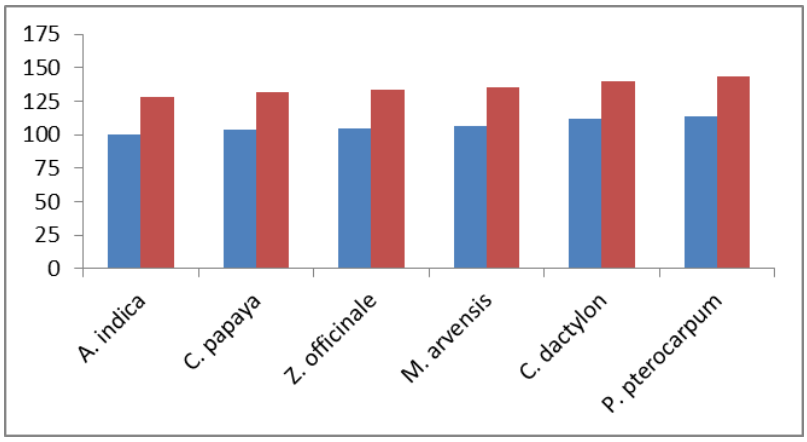


Fig 4.25 Comparative analyses MIC and MBC of six plants extract against the enteropathogenic bacteria

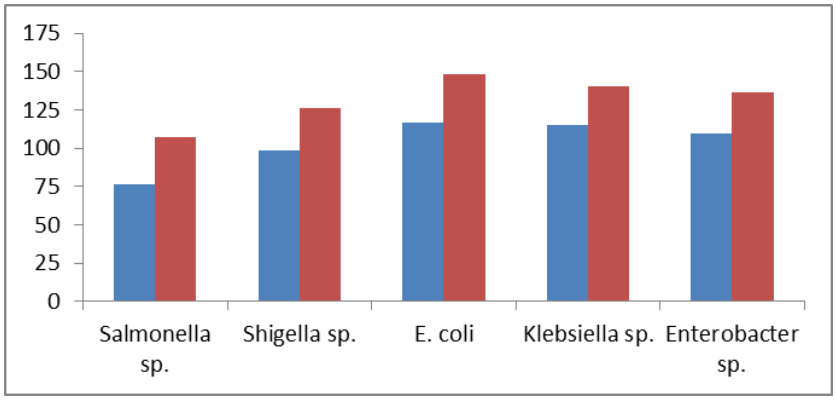


Fig 4.26 Comparison of MIC and MBC values against the enteropathogenic bacteria

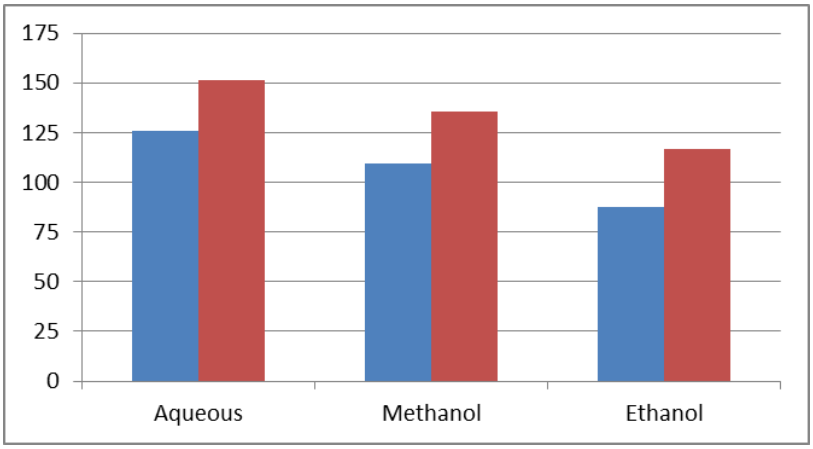


Fig 4.27 Comparison of three solvents against the enteropathogenic.

4.6 *In vivo* trail of plant extracts against the pathogenic enterobacteria of poultry.

The experiment was conducted to study the effect of *A. indica* leaves and papaya fruit extracts to control enterobacteria associated diseases of poultry. A total of 84, one day old Sonali chicks were purchased from a local hatchery (RMR Nourish Poultry & Hatchery Ltd.) and kept in experimental cages in 2 groups with three replications. The initial weight of each chick was measured. A weighted amount of the ration was feeded with pure water which was made available up to 15 days. After that the chicks were transferred into the pen according to the experimental design which is described in below:

Effect of *A. indica* leaves extract to control pathogenic enterobacteria

The aqueous extract of *A. indica* was directly ingested to the experimental poultry according to the treatments (Table 4.31 and Plate 4.13). From the results it was observed that the weight of the poultry was rapidly increased in TN_1 (Additive), TN_2 (Neem) and TN3 (Additive+Neem) treatments compared to others. On 45^{th} day, the maximum weight gain was observed in TN_3 (Add.+Neem) which was 11.16% higher than control. In a combination of extracts and inoculum treatments i.e. TN_4 (N+*Salmonella*), TN_5 (N+*Shigella*), TN_6 (N+*Enterobacter*), TN_7 (N+*E.Coli*) and TN_8 (N+*Klebsiella*)) weight gain were increased moderately and no disease symptoms were observed. But in case of single inoculum ingestion, all the poultry were dead after 5 days of ingestion in TN_9 (*Salmonella*); after 7 days in TN_{10} (*Shigella*) and TN11 (*Klebsiella*) (data not shown in table). Hundred percent mortality was observed in TN_9, TN_{10} and TN_{11} while the pathogenic bacteria *Salmonella* sp. *Shigella* sp. *and Klebsiella* sp. were ingested; *E. coli* sp. (TN_{12}) and *Enterobacter* sp. (TN_{13}) showed disease symptoms after 15 days and the rest of the poultry remain healthy.

Table 4.31 Average live weight and weight gain of Sonali chick in different treatments using *A. dirachta* leaf extract.

Treatments	Initial average live weight(g) at 15th day (w_1)	After 30 Days		After 45 Days		Symptoms	Mortality
		Average live weight(g) (w)	Average Weight gain (g) (w_2-w_1)	Average final live weight(g) (w_3)	Average Weight gain (g) (w_3-w_1)		
TNo (Control)	81.10	281.16e	200.06	510.20e	429.1	Healthy	Nil
TN_1(Additive)	86.90	292.73b	205.83	553.16b	466.26	Healthy	Nil
TN_2 (Neem)	84.13	288.5c	204.37	540.90c	456.77	Healthy	Nil
TN_3 (Add.+Neem)	90.75	304.6a	213.85	573.8a	483.05	Healthy	Nil
TN_4 (N+*Salmonella*)	72.1	254.50f	184.44	419.53f	347.43	Healthy	Nil
TN_5 (N+*Shigella*)	71.26	230.40ghi	159.14	416.16f	344.9	Healthy	Nil
TN_6 (N+*Enterobacter*)	73.43	237.76g	164.33	414.33f	340.9	Healthy	Nil
TN_7 (N+*E.Coli*)	72.93	228.30ghi	155.37	401.66gh	328.73	Healthy	Nil
TN_8(N+*Klebsiella*)	72.56	222.50hi	149.94	401.46gh	328.9	Healthy	Nil
TN_9 (*Salmonella*)	53.10	-	-	-	-	Dead	100%
TN_{10} (*Shigella*)	54.16	-	-	-	-	Dead	100%
TN_{11} (*Klebsiella*)	51.70	-	-	-	-	Dead	100%
TN_{12} (*E.Coli*)	53.53	172.53j	119	236.80i	183.27	Affected	Nil
TN_{13} (*Enterobacter*)	52.13	174.56j	122.4	233.90i	181.77	Affected	Nil

[Values in a column having the same letter did not differ significantly P= 0.05 according to DMRT]

Effect of *C. papaya* fruit extracts to control pathogenic enterobacteria

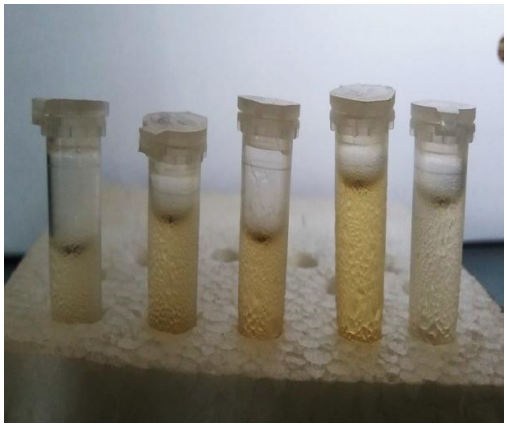
The aqueous extract of *C. papaya* was directly ingested into the experimental poultry according to the treatments (Table 4.32 and Plate 4.13). From the results it was observed that the weight of the poultry was rapidly increased in TP_1 (Additive), TP_2 (Papaya) and TP3 (Additive+Papaya) compare to others. On 45^{th} day, the maximum weight gain was observed in TP_3 (Additive +Papaya) which was 11.37% higher than control. In a combination of extracts and inoculum namely as TP_4 (P+*Salmonella*), TP_5 (P+*Shigella*), TP_6 (P+*Enterobacter*), TP_7 (P+*E.Coli*) and TP_8 (P+*Klebsiella*) weight gain were increased moderately and no disease symptoms were observed. But in the case of only inoculum ingestion, the poultry was dead after 5 days in TP_9 (*Salmonella*); after 7 days in TP_{10} (*Shigella*) and TP_{11} (*Klebsiella*) (data not shown in table). Hundred percent mortality was observed in TP_9, TP_{10} and TP_{11} treatment while the pathogenic bacteria *Salmonella* sp. *Shigella* sp. *and Klebsiella* sp. were ingested; *E. coli* sp. (TP_{12}) and *Enterobacter* sp. (TP_{13}) showed disease symptoms after 15 days and the rest of the poultry remain healthy.

Table 4.32 Average live weight and weight gain (g) of sonali chicks in different treatments using papaya fruit extract

Treatments	Initial average live weight(gm) at 15th day(w_1)	After 30 Days		After 45 Days		Symptoms	Mortality
		Average live weight(gm) (w_2)	Average Weight gain (gm) (w_2-w_1)	Average final live weight(gm) (w_3)	Average Weight gain (gm) (w_3-w_1)		
TPo (Control)	81.10	281.16e	200.06	510.20e	429.1	Healthy	Nil
TP_1 (Additive)	86.90	292.73b	205.83	553.16b	466.26	Healthy	Nil
TP_2 (papaya)	83.4	283.46de	200.06	523.26d	439.86	Healthy	Nil
TP_3 (Add.+Papaya)	85.7	301.46a	215.76	569.9a	484.2	Healthy	Nil
TP_4 (P+*Salmonella*)	71.53	232.63gh	161.1	398.93gh	327.07	Healthy	Nil
TP_5 (P+*Shigella*)	72.50	230.76ghi	158.26	405.23g	332.5	Healthy	Nil
TP_6 (P+*Enterobacter*)	74.03	223.16hi	149.13	392.73h	318.67	Healthy	Nil
TP_7 (P+*E.Coli*)	69.56	221.43i	151.87	393.93h	324.37	Healthy	Nil
TP_8 (P+*Klebsiella*)	74.03	174.70j	100.67	396h	321.97	Healthy	Nil
TP_9 (*Salmonella*)	53.10	-	-	-	-	Dead	100%
TP_{10} (*Shigella*)	54.16	-	-	-	-	Dead	100%
TP_{11} (*Klebsiella*)	51.70	-	-	-	-	Dead	100%
TTP_{12} (*E. Coli*)	53.53	172.53j	119	236.80i	183.27	Affected	Nil
TP_{13} (*Enterobacter*)	52.13	174.56j	122.4	233.90i	181.77	Affected	Nil

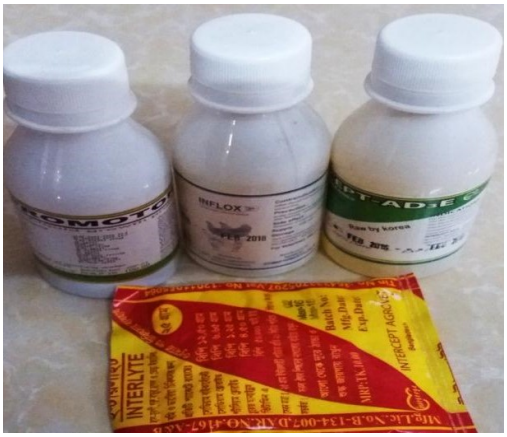
[Values in a column having the same letter did not differ significantly P= 0.05 according to DMRT]

Poultry feed



Bacterial inoculum

Plant extracts



Additives

Plate 4.11 Photographs showing poultry feed, bacterial inoculum, plant extracts and additives used in *in vivo* condition.

Experimental farms

Plate 4.12 Photographs showing experimental farms and measuring of treated chicken.

Control (TP_0)

A. indica **(TN_4-TN_8)**

C. papaya **(TP_4-TP_8)**

Only pathogen (TN_9-TN_{13})

Plate 4.13 Photographs showing chicken under control and treatment

CHAPTER FIVE
DISCUSSION

Poultry farming is the most prosperous and uprising sector in Bangladesh and many people are involved with it for their livelihood and have great extent in our national economy. It is a good source of nutritious animal protein and supplies 22-27% of the total animal protein in the country. But the poultry flocks are affected by different diseases due to poor hygiene and sanitation system, lack of consciousness of the poultry farmers and workers, microbial contamination of the feed, cross-contamination of the poultry, etc. Besides, different additives and antibiotics are massively used in poultry nourishing which is cost-effective and risk to man and the environment. Thus the present perspective research has been conducted with the different subheadings which are given below:

Survey

In the present study, twenty poultry farms at different locations of Rajshahi Metropolis and surrounding areas were surveyed from January to December 2013. The farms were mainly surveyed to assess their sanitation condition and hygienic status. The survey report reveals that coccidiosis, avian influenza, bacillary white diarrhea, mycoplasmosis; gamburo, ranikhet and peritonities (milky fluid) etc. were common diseases in the poultry of the studied areas. The most common bacterial diseases of poultry are found Ecolisis, Salmonelloses, Paratyphoid, Fowl cholera, Riemerella anatipestifer infections, Mycoplasma, Necrotic enteritis, Cholangiohepatitis etc. (G. Lorenzoni, 2010)

The gastrointestinal tract is the place of residence and transit of pathogenic and non-pathogenic microorganisms and a major portal of entry for many pathogens. Bacterial contamination of food causes a wide range of gastrointestinal diseases (GIDs). The common GID are *Bacillus cereus* infection, *Shigella* dysentery, Cholera, *Campylobacter* gastroenteritis, Typhoid fever, Yersinia infection, Salmonellosis, *E. coli* infection, peptic ulcers etc (G. Lorenzoni, 2010). In this prospective study, the main intention was in pathogenic enterobacteria thus the GID was monitored. The Gastrointestinal Disease (GID) incidence was varied with the month. The ranges of GID incidence were 50.0 to 78.57%. The highest gastro- intestine disease (GID) was recorded in January and the lowest was in April. The

prevalence of Newcastle Disease (ND) was found to be the highest in the broiler (7.85%), followed by Fowl typhoid (6.58%), Mycoplasma (5.68%), Escherichia coli (5.52%), Coccidiosis (4.59%) and Mycotoxicosis (4.56%), Abbas et al. (2015).

Temperature and relative humidity are the main factors for the growth and disease development of poultry. From the survey results, it was observed that January was the coolest month while comparatively minimum temperature and RH (%) was measured and maximum disease incidence was observed. The minimum DI (%) was recorded in July where temperature and relative humidity was high. So, it seems that the disease incidence was directly correlated with temperature and relative humidity. In earlier Abbas et al. (2015) indicated that the period between April and June tended to be comparatively healthier for broilers because of the low incidence of disease at this time.

In the case of hygiene and sanitation practices, the cleanness status of the farms was good and they cleaned the farms regularly. Only fifty-five percent of farms used sanitizer like timsen, glutex, lifeline etc. The room conditions of most of the farms were good with good ventilation and sufficient light. Besides, sixty percent of farms were used tube-well water and none of the farms used purified water. Folorunso et al. (2014) examined water troughs with the minimal bacterial load when cleaned daily, those left uncleaned for 3, 5 and 7 days had increasingly high bacterial loads. Hence water is the source of many pathogens so farmers should care about the use of water.

From the survey results, it was also observed that most of the poultry workers were not aware of their hygiene. They did not follow any precautions while coming in and out of the pen. Most of the poultry workers were not strictly followed any rules and regulations for sanitation and hygiene and only fifteen percent of farm' workers scored excellent in this. Musa et al. (2013) suggest strong policy measures to change the attitude of poultry farmers and to increase awareness of the effects of potential outbreaks of poultry disease in Nigeria.

The waste management systems (liquid and solid litter) of the poultry farms in Rajshahi city were not satisfactory. In the case of liquid litter disposal, twenty percent of the poultry farms did not follow any waste disposal system whereas fifty-five farms scored 'Good' and twenty--five farms scored 'very good'. All poultry farms scored 'very good' in solid litter disposal. Thus more attention is needed to improve the waste disposal system and good farming practices.

Microbial quality assessment of poultry feed samples

Microbiological quality assessment is an integral part of any product development as it gives a clue to the safety and keeping quality of the feed. One of the microbiological metrics for food quality is the total viable number of aerobic mesophilic microorganisms found in food. Aerobic organism activity demonstrates the nature of favorable conditions for microorganism multiplication. From the results, it was observed that the ranges of cfu of total aerobic plate count (APC) of poultry feed samples were recorded as 0.28×10^4 to 2.8×10^8/gm while indicating the poor sanitary quality of the feed, because in case of sanitary quality assessment $>10^6$cfu/gm considered as hazard condition.

A large number of coliform was recorded in poultry feed samples and the ranges of total coliform were 0.1×10^4 to 9.6×10^4 /gm. The highest cfu was at Peyarul poultry farm and the lowest was at Mostak poultry farm. The ranges of cfu of *E. coli* were 0.0 to 1.80×10^4/gm. The highest cfu was counted at Juwel poultry farm and the lowest was counted a at Roshid and Ahab poultry farms. These findings also support other studies which gave total plate count, total coliforms and *E. coli.* (Higenyi, et al. 2014).

For *Salmonella-Shigella* the ranges of cfu were recorded as 0.0to 0.85×10^4/gm. The highest cfu was counted at Juwel poultry farm and the lowest was counted at Peyarul and Mostak poultry farms. From the results it was exhibited that all the feed samples of the poultry farms of Rajshahi Metropolis were highly

contaminated with coliforms and other bacteria. In an earlier study, Tabib et al. (1998) analyzed seemingly normal feed and feed ingredients and reported coliform numbers ranging from 5 to 9.1×10^5/gm. De-Shalom (1999) investigated the bacterial contaminants associated with commercial poultry feeds and reported *Staphylococcus aureus* as the most predominant bacterial organism with 52 cfu/gm, followed by *Salmonella typhi* with 48 cfu/gm, *Bacillus cereus* 40 cfu/gm and *Pseudomonas aeruginosa* 18 cfu/gm.

Isolation and identification of enterobacteria

Total forty-four enterobacterial isolates were isolated by using three selective media namely MacConkey agar, EMB agar, and SS agar. The characteristics of colony morphology, gram staining reactivity, and microscopic features were recorded. All the isolates were gram-negative according to the results of gram staining. Further biochemical tests of the strains were conducted, i.e. (Motility test, Catalase test, Citrate test, Indole test, Starch hydrolysis test, Phenylalanine test, MR test, VP test, Oxidase test and KIA test). Selected enteropathogenic bacteria have been classified according to colony morphology, microscopic characteristics, and biochemical characteristics that have been validated by Micro-Rao online software. Finally, the forty-four isolates were identified as seven genera i.e. *Escherichia coli* (with 99.91% probability), Klebsiella sp. (with 100% probability), *Salmonella* sp. (with 93.2% probability), *Shigella* sp. (with 94.12% probability), *Enterobacter* sp. (with 99.03% probability), *Citrobacter* sp. (with 99.93% probability), and *Proteus* sp. (with 100% probability).

Pathogenicity test of isolated enterobacteria

Pathogenicity tests of the isolates were carried out by hemolytic activity test, virulence test, and hemagglutination test. Twelve of the 44 isolates showed positive hemolytic activity on blood agar because these isolates break down red blood cells and result in a clear zone around the colony. Isenberg (1992) reported similar findings.

In the virulence test, the twelve hemolytic positive bacteria were subjected and directly tested in poultry. Out of twelve isolates, three isolates showed highly toxic reactivity and died after 6 to 9 days, five isolates showed severe sickness symptoms while three isolates showed little illness. ISE-4 showed watery stool symptoms. Similar findings were reported by Stelma et al. (1987).

The Rose Bengal Agglutinin test (RBAT) is a rapid agglutinin assay for screening of animal sera (Cattle, sheep, and bird) (Cernyceva et al. 1977). Various authors have found that its sensitivity is fairly high and that its result agrees with those of other serological tests in as many as 99.1% of cases. Thus a study of the mechanism of the RBAT has drawn attention to the considerable influence of the activity of antigen on the intensity and specificity of the test. Some data show that IgG antibody plays an active part in the Rose Bengal agglutination test (Levieux, 1974, Morgan, 1971). This information has been of great importance in developing serological test and makes RBAT the ideal test for small laboratories. Rose Bengal Agglutination Test (RBAT) was carried out for pathogenic strain detection in this study. It was reproached from the results that the RBAT antisera agglutination value ranged from weak (++) to high (++++). At the first fold, out of 12 only11 antisera showed positive agglutination and only 6 reacted strongly (++++). At the second fold, 9 antisera showed positive and among them 6 reacted strongly (++++). In the case of the 3rd and 4th fold, out of 12, only 6 antisera reacted strongly (++++) and others showed negative results. From the results, it was remarkably noticed that with the increases of serum dilution the specificity also improved and showed distinct agglutination reactivity. Roman et al. (2011) also reported that RBAT was highly sensitive and a simple adaptation to test serum dilution improve its specificity and considerably reduce for additional serological tests which completely support the present finding. This result also supports the clinical sign of poultry in an experimental study.

Finally, based on pathogenicity test results, five isolates considered as pathogenic enterobacteria namely *Escherichia coli* (isolate code ISE-4), *Klebsiella* sp. (isolate code IGM-32 and BSE-38), *Enterobacter* sp. (isolate code

JSE-2), *Salmonella* sp. (isolate code JSS-14) and *Shigella* sp. (isolate code MGS-17)

***In vitro* evaluation of six plants extracts against entero-pathogenic bacteria**

In recent years, plant antimicrobial properties have been reported and interest continues to this day (Domans and Deans, 2000; Radhakrishan-Sridhar and Velusamy-Rajaopal, 2003). The plant extracts which proved to be effective can be used as natural alternative preservatives to control food-borne pathogens for avoiding health hazards chemical antimicrobial agent (Mostafa et al. 2017). Plants synthesize secondary metabolites and many compounds with complex molecular structures and some of them have been related to antimicrobial properties found. So in this context, six medicinal plants were tested against the selected enterobacteria to test their antibacterial efficacy and along with this their phytoconstituents were also analyzed.

Investigation of phytochemistry of the studied plant extracts

Phytochemical components depend on polarity and scale, which are important factors in the isolation of individual compounds. Therefore, the selection of solvents for phytocompound extraction is the key to success. Resistance to antibiotics has become a global issue. Multiple tolerances in human pathogenic microorganisms have increased in recent years, largely due to the indiscriminate use of synthetic antimicrobial drugs widely used in infectious disease care (Westh et al. 2004). This has forced scientists from various sources such as medicinal plants to search for new antimicrobial substances. Screening of many plant families should continue the search for new antibacterial agents (Parekh and Chanda, 2007). Therefore, the selection of solvents for phyto-compound extraction is the key to success. Tiwari et al. (2011) proposed the use of aqueous solvents to remove anthocyanins, starch, tannins, saponins, polypeptides and lectins. Of tannins, polyphenols, polyacetylene, flavonol, terpenoids, sterols and alkaloids, ethanol is stronger. On the other hand, methanol is good for anthocyanins, terpenoids, saponins, tannins, xanthoxyllins, totarol, lactones, quassionoids, flavones, phenones and polyphenols while acetone is stronger for phenols and

flavonols. From the experimental extracts of six plants namely *A. indica*, *C. papya, Z. officinale, C. dactylon, M. arvensis* and *P. pterocarpum* were tested against the selected pathogenic enterobacteria and ethanol, methanol and aqueous were used as solvent. Most of the plant extracts showed positive results for alkaloid, tannin, saponin, steroid, and glycoside. In earlier studies flavonoid, tannin, glycoside, steroid, saponin, and phenolic compounds in plant samples support the present results. Spot phytochemical studies (qualitative test) of current research work proved that all studied plant parts containing more or less alkaloid, flavonoid, glycoside, saponin, phenol, tannin, terpenoid, and steroid, and are well known for antibacterial activities (Tiwari et al. 2011).

High-performance liquid chromatography (HPLC) is the modern technique for the analysis of individual components in plant extracts. Supe and Daniel (2015) clarified that HPLC is a simple, accurate, and selective technique for separation and quantification of the identified compound. In the present study, six plant extracts were selected for HPLC analysis. From the screening of HPLC results and peak profiles of the extracts, the highest number of phyto-compounds (30) found in *A. indica*,. Next of the number of phytochemicals found as *C. papaya* (27), *P. pterocarpum*(19), and *Z. officinale* (15) and *M. arvensis* (14) and lowest in *C. dactylon* (12). HPLC reported that β-sitosterol is an effective antibacterial agent (Joy et al. 2012 and Sen et al. 2012). Lots of finding of antibacterial compound like caffeic acid (Wild 1994), cysteine (Garcia et al. 1998, Pelegrini et al. 2011, and Salas et al. 2015), gallic acid (Oyedeji et al. 2014), glycine (Minami et al. 2004) which are also well known bio-active and antibacterial compound that can inhibit or kill the selected pathogenic bacteria.

The HPLC results reveal that *A. indica* leaves extract contained oxalic acid, ascorbic acid, gallic acid, glycine, cysteine , alanine , tryptophan , threonine , vanilic acid, phenilalanine, leusine, and citrate.

The HPLC results revealthat *C. papaya* fruit extract contained ascorbic acid, β-sitosterol, glycine, cysteine, vanillic acid, caffeic acid, syringic acid, phenylalanine, p-coumaric acid and citrate.

The HPLC results reveal that *Z. officinale* rhizome extract contained Oxalic acid, Serine, tryptophan, Catechin, Phenilalanine, Leusine, Methinine, and citrate.

The HPLC results reveal that *M. arvensis* leaves extract contained Oxalic acid, Serine, tryptophan, threonine, Phenylalanine, Leusine and citrate.

The HPLC results reveal that *P. pterocarpum* bark extract contained Oxalic acid, Serine, glycine, Alanine, tryptophan, threonine, Leusine, Methinine, and citrate.

The HPLC results reveal that *C. dactylon* leaves extract contained Oxalic acid, Serine, tryptophan, threonine, Phenylalanine, Leusine and citrate.

Phytochemicals were also detected from papaya fruit by earlier scientists such as ascorbic acid (Laura et al. 2011); β-sitosterol (Oloyede et al. 2011); glycine and cysteine (Rincy and Rajani 2017); vanillic acid (Mojisola et al. 2008); caffeic acid, syringic acid and p-coumaric acid (Laura et al. 2011) which partially support the present finding. Besides this, Julianti (2014) identified the active fractions of *C. papaya* fruit in methanol extract by HPLC-based profiling and isolated a total of nine compounds, among them, four were known as flavonols-manghaslin, clitorin, rutin and nicotiflorin and five were unknown compounds. Wang et al. (2015) isolated campaign from *C. papaya* using HPLC method. Maisarah et al. (2013) showed that papaya fruits exhibited the high amount of ascorbic acid, β-carotene content and vitamin E (85.60, 3.86 and 4.09 mg/100 g, respectively) by using HPLC. Presence of these 13 reported and well known antimicrobial phytochemicals was checked among the studied six plant extracts. Results revealed that some of them presence in each studied plant extract. The analysis therefore indicated that the presence of these compounds in the plant extracts

studied may contribute to their potency to inhibit the growth of the pathogenic enterobacteria selected.

***In vitro* assessment of six plant extracts antibacterial activities**

Antibiotics, drugs and active phytochemicals are chemicals that either kill bacteria directly (bactericidal) or hamper their ability to grow and reproduce (bacteriostatic). These chemicals, particularly phytochemicals, stop or interfere with a number of daily cellular processes that bacteria depend on for their growth and survival, such as: crippling bacterial cell wall development that protects the cell from the external environment, interfering with protein synthesis by binding to protein-building machinery or amino acid, Metabolic havoc or blocking of DNA and RNA synthesis (Kohanski et al. 2010). The antibacterial compound also disrupting membrane potentiality and integrity, and induced lysis of cell wall, blebbing and leakage of cellular contents, or inhibition of macromolecular synthesis (Zasloff 2002). It would be the best choice to combine antibiotics with plant extracts or active principles of herbal products that are non-toxic to mammalian cells. Therefore, assessing the antibacterial activity of extracts obtained from medicinal plants may help devise alternative or combinational treatment approaches for the treatment of entropathogenic bacteria infections.

In the present investigation methanol, ethanol and aqueous extract were derived from six plants such *Azadirachta indica*, *Carica papya, Zingiber officinale, Cynodon dactylon, Mentha arvensis* and *Peltophorum pterocarpum* and their efficacy were tested against the five selected pathogenic bacteria e.g. *Escherichia coli* sp., *Klebsiella* sp., *Shigella* sp., *Salmonella* sp. and *Enterobacter* sp. However, the degrees of antibacterial activities of studied extracts were varied. Such variations may be attributable to the differences in the chemical composition of these extracts since the plant's secondary metabolites have many effects including antibacterial properties (Noumedem et al. 2013 and Cowan 1999).

A. indica leaves extracts

The results of the antibacterial assays showed that *A. indica* leaves extracts is potent to a *Salmonella* sp, *Shigella* sp., *Klebsiella* sp, *E. coli* and *Enterobacter* sp. But the inhibition zones were varied at different applications. Assessed against Shigella sp, the highest inhibition region for ethanolic extracts of *A. indica*. The highest inhibition zone for ethanol leaf extracts was assessed against Shigella sp. The maximum inhibition zone against Shigella sp was reported as a result of aqueous leaf extracts. Timothy et al. (2011) experimented with the dose-dependent antibacterial activity of the extract (6.25 mg, 12.5 mg, 25 mg, 50 mg and 100 mg) at the doses tested on *Escherichia coli, Staphylococcus aureus* and *Salmonella typhi*. The ethanolic extract of *A. indica* was studied by Prashar et al. (2012). High potential against Gram-positive bacteria. Francine et.al (2015) investigated the in vitro effect on *Staphylococcus aureus* and *Escherichia coli* of extracts of different neem plants (leaf and bark), compared neem leaves and neem barks and observed that fresh extracts were always found to be more productive than dry extracts; in all cases, the same applies to ethanol extracts as to aqueous extracts. Rasool et al. (2017) evaluated the antimicrobial activity of ethanolic extracts of *A. indica* and *Psidium guajava* against four clinically important bacteria namely *Staphylococcus aureus, Salmonella typhi, E. coli* and *Pseudomonas aeruginosa* exhibited zone of inhibition against all isolated bacteria.

In the phytochemical spot test results proved the presence for alkaloid, flavonoid, saponin, tannin and steroid, while HPLC analysis detected ascorbic acid, ascorbic acid, glycine, cysteine, vanillic acid, phenylalanin and citrate in *A. indica* leaf extract. These phytochemicals are very toxic to bacteria that support the antibacterial activity of the plants.

Carica papaya Fruits Extract

Aqueous, methanol and ethanol extracts of *C. papaya* fruits were investigated for their antimicrobial activity. The methanolic extract showed the widest inhibition zone (14.8 mm) against *Shigella*. Anthonia and Olumide (2010) also observed more effective results in methanolic extract of *C. papaya* fruits than ethanolic

extract against *Salmonella* sp. Osato et al. (1993) determined the zone of inhibition 13.5, 8.0 and 10.5mm against *E coli*, *Salmonella* sp and *Klebsiella* sp., respectively when they investigated on antimicrobial activities of unripe papaya. Doughari et al. (2007a) reported the inhibition zone 8 and 14 mm against *E. coli* and *Salmonella* sp, respectively for antibacterial activity of *C. papaya*. Anibijuwon and Udeze (2009) researched the function of *C. papaya* antibacterial. The *C. papaya* against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus* and *Proteus mirabilis* showed the highest inhibition (14 mm) of *Pseudomonas aeruginosa*, while the aqueous extract displayed a greater inhibition of higher bacterial activity than the organic solvent. Papaya fruit has been reported to contain alkaloids, flavonoids, steroids, saponin, terpenoid, sterol, tannin, and steroids, suggesting the *C. Papaya* have the potential to produce drugs against pathogens that cause infections of the urinary tract (Yusha'u et al. 2009, Dada et al. 2016, and Seow et al. 2016). Ocloo et al. (2012)'s investigation into aqueous and organic extracts of dried papaya pulp confirmed active compounds and antibacterial activity against *Staphylococcus aureus, Escherichia coli and Shigella flexneri*. Dawkins et al. (2003) studied *C. papaya* antibacterial activity and observed the inhibition zone on growing wound species.

The phytochemical spot test results proved the presence of alkaloid, flavonoid, saponin, tannin, and steroid, while HPLC analysis detected ascorbic acid, β-sitosterol, glycine, cysteine, vanillic acid, caffeic acid, syringic acid and p-coumaric acid in *C. papaya* fruit extract that supports the antibacterial activity of the plant.

***Z. officinale* rhizome extract**

Methanol, ethanol and aqueous extracts of the rhizome of *Z. officinale* plant were used for their antimicrobial activity against the selected pathogen. The maximum region of *shigella sp.* inhibition. The methanolic extracts displayed *Klebsiella sp's* highest inhibition zone. Njobdi et al. (2018) reported that *Z. officinale* both fresh and dried extracts inhibit the growth of *S. aureus* and *E. coli* similar to some standard antibiotics.

The phytochemical spot test results proved the presence for alkaloid, flavonoid, saponin, tannin and steroid, while HPLC analysis detected oxalic acid, phenylalanine and citrate in *Z. officinale* extrac. These phytochemicals are very toxic to bacteria that support the antibacterial activity of the plant.

***M. arvensis* leaves extract**

The findings of the antibacterial activity assays showed that the inhibitory activity against the identified pathogen was shown by this investigated plant, but the inhibition zone was varied at different concentrations. The ethanol extracts display the highest inhibition zone against E. coli. Johnson et al. (2011) determine the anti-bacterial efficacy of internodal chloroform, ethanol, ethyl acetate and water extracts from *Mentha arvensis* calli extracts to *Salmonella typhi, Streptococcus pyogenes*, *Proteus vulgaris* and *Bacillus subtilis*. Ling Zhang et al. (2015) followed tests for disc diffusion and microdilution to determine the extract's antibacterial activity by measuring the inhibition region.

The phytochemical spot test results proved the presence of flavonoid, saponin and steroid while HPLC analysis detected oxalic acid, phenylalanine and citrate in *M. arvensis* leaf extract. These phytochemicals are very toxic to bacteria and support the antibacterial activity of the plant.

***P. pterocarpum* bark extract**

It was reported that the *P. pterocarpum* bark extracts had the inhibitory potentiality against the selected pathogens. The methanol extracts showed the highest zone of inhibition against *Shigella* sp. In an earlier study Kumar and Rathinam (2009) reported that among the three plants, *P. pterocarpum* hexane extract exhibited maximum region against *K. pneumoniae* at concentration of 225 µg/mL and minimum inhibition region observed in *C. racemosa* and *B. purpurea* at 75 µg/ml against organism *E. coli*, respectively. Jagessar (2007) reported that ethanol and ethyl acetate extracts of *Luffa perculata* and *Peltophorum pterocarpum* can be used as herbal medicines in the control of *E. coli and S. aureus* induced medical diseases, following clinical trials.

***C. dactylone leaves* extract**

The results of the antibacterial activity assays indicated *C. dactylone leaves* extracts showed inhibitory activity against the sleeted pathogens. The ethanol extracts recorded that the maximum inhibition zone against *Shigella* sp. Abdullah et al. (2013) researched anti-bacterial pathogen's antimicrobial activity from extracts of ethanol and ethyl acetate. Extract of methanol and acetone displayed activity to *B. cereus* (8.0 ± 0.0 mm) and *B. subtilis* (7.0 ± 0.0 mm), while chloroform extract demonstrated activity to *B. subtilis* (7.0 ± 0.0 mm) and *S. pyogenes* (8.3 ± 0.6 mm), respectively. For aqueous leaf extracts, the highest zone of inhibition against *Shigella* sp. Rahman (2014) reported that aqueous extract of Cynodon dactylon's entire plant may be considered as an antibacterial agent and may be used as a source of antibiotic substances to treat bacterial infections.

The phytochemical spot test results proved the presence for flavonoid, saponin, tannin and steroid, while HPLC analysis detected oxalic acid and citrate in *C. dactylone leaves* extract. These phytochemicals can play a great role in controlling the bacteria.

From the results, it reveals that among the selected six plant extracts, *C. papaya* fruit extract was the best for creating an inhibition zone against selected pathogenic bacteria and ethanol was the best solvent. From the result, it also found that *E. coli*, *Shigella* sp. *Klebsiella* sp. and *Enterobacter* sp. more sensitive to *C. papaya*. The rank of the zone of inhibition of the plant extracts was found as *C. papaya >A. indica>Z. officinale>M. arvensis>C. dactylon>P pterocarpum* and the rank for solvent as Ethanol > Methanol > Aqueous.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

In microbiology, minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism. Confirming the resistance of microorganisms to an antimicrobial agent and monitoring the development of new antimicrobial agents is essential in diagnostic laboratories. A lower MIC is a better signal for antimicrobial. In general, the most basic laboratory measurement of the action of an antimicrobial

agent against an organism is called a MIC. Clinically, the minimum inhibitory concentrations are used not only to determine the number of antibiotics to be received by the patient but also the type of antibiotic used, which in turn reduces the chance of microbial resistance to specific antimicrobial agents (Turnidge et al. 2003).

The lowest concentration of antibiotic required to kill a particular bacterium is the minimum bactericidal concentration (MBC). The minimum inhibitory concentration (MIC) is not as commonly seen. Through subculturing to agar media without antibiotics, it can be determined from broth dilution MIC measurements. Antimicrobials are generally considered bactericidal when the MBC is no more than four times the MIC (French 2006).

From the results, all the plant extracts tested showed antibacterial activity against selected pathogenic bacteria on a large or small scale. The minimum values of six plants for inhibitory concentration (MIC) ranged from 50 to 150 mg/ml and minimum bactericidal concentration (MBC) values were ranged from 75 to 175 mg/ml. These differences could be due to the differences in the chemical composition of these extracts as the secondary metabolites of plants are deferred from plant to plant.

MIC and MBC for *A. indica*

In the present investigation MIC and MBC of *A. indica* was investigated. The ethanol extract showed maximum activity with MIC (50-125 mg / ml) and MBC (75-150 mg / ml) values, while the methanol extract displayed mild MIC (50-125 mg / ml) and MBC (75-150 mg / ml) values and aqueous extract's MIC levels were 75-150 mg / ml and MBC values were 100-175 mg / ml. Timothy et al. (2011) experimented with the minimum inhibitory concentration (MIC) for. The minimum inhibitory concentration (MIC) for *E. coli, S. aureus* and *S. typhi* were 2.39 mg, 3.31 mg and 4.79 mg respectively were experimented with by Timothy et al. (2011). The effect of 100 mg (18 mm) ethanolic leaf extract was statistically significantly higher ($p<0.05$) than that of 1.5 mg (14 mm) ciprofloxacin on S.

typhi. Prashar et al (2011) determined 0.87 mg/ml and 1.74 mg/ml for ethanol extract of MIC and MBC value against *S aureus*; for *Bacillus subtilis* 0.36 mg/ml and 0.72 mg/ml; and for *E coli* were 1.26 mg/ ml and 2.52 mg/ml, respectively.

MIC and MBC for *Carica papaya*

In the present investigation MIC and MBC of *C. papaya* was investigated. The ethanol extract showed maximal activity with MIC values (50-125 mg / ml) and MBC values (100-150 mg / ml), while the methanol extract displayed modest MIC values (50-125 mg / ml) and MBC values (75-150 mg / ml), and the MIC value levels for aqueous extract were 100-150 mg/ml, and the MBC values were 125-150 mg/ml. Osato et al. (1993) investigated on determining minimum inhibitory concentration (MIC) of papaya as 500mg/ml against used bacterial strains. The papayas MIC and MBC were investigated by Doughari et al. (2007a). Extracts of papaya ranged from 50-200 mg/ml to pathogenic bacteria. MIC of *C. papaya* was determined by Akujobi et al. (2010), papaya fruit extracts were reported to be the most susceptible to *Staphylococcus aureus* with a minimum inhibition concentration (MIC) of 1:64 while *Enterococcus faecalis* was the least sensitive. Hema et al. (2013) studied MIC of *C. papaya* against ten clinical pathogens and reported that the MIC and MBC value of *Carica* was 3.175 and 6.25 µg/ml, respectively. The MIC meaning of *Carica* fruit extract was stated by Aguoru et al. (2015) as 12.5 mg/ml as 12.5mg/ml against both *Salmonella* sp. and *Shigella* sp. and 25mg/ml against *E. coli* while MBC value for both *E. coli* and *Shigella* sp. were determined 200mg/ml and for *Salmonella* it was 100mg/ml.

MIC and MBC for *Z. officinale*

In the present investigation MIC and MBC of *Z. officinale* was investigated. The ethanol extract displayed maximum activity with MIC values (50-100 mg / ml) and MBC values (75-150 mg / ml), while the methanol extract showed mild MIC values (100-150 mg/ml) and MBC values (125-150 mg / ml) and ranges of aqueous extract MIC values were 50-150 mg / ml, and MBC values were 125-175 mg / ml, respectively. Similar results against *S. aureus* and *E. coli* were reviewed by Njobdi et al. (2018).

MIC and MBC for *M. arvensis*

In the present investigation MIC and MBC of *M. arvensis* was investigated. The ethanol extract displayed maximum activity with MIC values (50-100 mg / ml) and MBC values (75-125 mg / ml), while the methanol extract showed moderate MIC values (75-150 mg/ml) and MBC values (125-150 mg / ml) and ranges of aqueous extract MIC values were 125-150 mg/ml and MBC values 125-175 mg / ml. Zhang et al. (2015) assess d the extract's antibacterial activity by measuring the inhibition region.

MIC and MBC for *C. dactylone*

In the present investigation MIC and MBC of *C. dactylone* was investigated. The ethanol extract displayed maximum activity with MIC values (75-125 mg/ml) and MBC values (100-150 mg / ml), while the methanol extract showed mild MIC values (100-125 mg/ml) and MBC values (125-150 mg / ml) and 125-150 mg / ml MIC values and 150-175 mg / ml MBC values. Marasini (2015) *C. dactylon, C. camphora, C. orchioides,* and *C. long* a plant extracts displayed a potential antibacterial activity of MIC <100μg/ml. Kalee swaran B. (2010) Minimum inhibitory concentrations (MIC) value of the ethanolic extract was response between in the range of 125 -62.5 µg/ml.

MIC and MBC for *P. pterocarpum*

In the present investigation MIC and MBC of *P. pterocarpum* was investigated. The ethanol extract showed maximum activity with MIC values (50-125 mg / ml) and MBC values (75-150 mgml-1), while the methanol extract showed moderate MIC values (75-150 mg / ml) and MBC values (125-175 mg / ml), and the MIC value ranges for aqueous extract were 100-150 mg / ml, and the MBC values were 150-175 mg / ml. The nearly similar result is found by A. Ravikumar and K. M. Subbu Rathinam (2009), Sukumaran S et al. (2011).

***In vivo* trial of neem leaves and papaya fruit extracts to control entero-bacteria associated diseases in poultry.**

Ethno-veterinary medicine (EVM) or ethnoveterinary medicine is a systematic, interdisciplinary study of local knowledge and related skills, traditions, values, practitioners and social structures related to healthcare and healthy food, employment and other income-producing animals. It plays a great role in a developing country where allopathic veterinary medicines are not accessible to the livestock producer. For many days, African and other countries use medicinal plants in poultry treatment. The use of conventional medicinal plants for primary health care has steadily increased throughout the world in recent years. So, increased attention on EVM is justified because it can reduce the excessive application of antibiotic and cost in poultry production as well as the practices will be eco-friendly.

In this study *A. indica* leaves and papaya fruit extracts were used to control entero-bacterial associated diseases of poultry for the development of EVM. The experiment was conducted at a commercial poultry farm. The experimental poultry was divided into two groups for neem and papaya separately with three replications.

This result reviled that, among the *A. indica* leaves extract treatments TN_3 (Add.+Neem) showed the best result in weight gaining and no disease symptoms have appeared. In a combination of extracts and inoculum (TN_4 -TN_8) weight gain was increased moderately and no disease symptoms were observed. But in the case of single inoculum ingestion (TN_{10} and TN_{11}), a hundred percent mortality was observed.

Papaya is one of the great sources of producing different types of metabolism which are very helpful for the antimicrobial and digestive intestinal system. In the case of papaya fruit extract among the treatments TP_3 (additive + papaya) showed the best result in weight gaining and no disease symptoms have appeared. But in the case of only inoculum ingestion (TP_9, TP_{10} and TP_{11}) hundred percent

mortality was observed. The use of Neem and Papaya drinking water extracts showed a greater increase in the bird's live weight compared to control. (Samanta and Dey, 1991). The plant extract contributes to the antibacterial properties of food supplements, resulting in better absorption of the nutrients found in the intestine and eventually contributing to weight gain change. The continuous treatment with neem leaves and papaya fruit extract produced a significant increase in the live body weight and a significant effect on antibacterial activity. It is concluded that ingestion of neem leaves and papaya fruit extract significantly increased live body weight and positively affected antimicrobial parameters as compared to that of a control group of chicken. So the effect of *A. indica* leaves and *C. papaya* fruit extracts to be an important source in EVM practices to control entero-bacterial diseases of poultry.

Also, herbal neem leaf extract and papaya fruit extract improved the weight gain of the poultry which is 11.16% and 11.37% higher than control, respectively. In earlier, it was reported that in broilers, the highest weight gain was observed when neem and papaya extract were used as water supplemented Meraj (1998). So, neem and papaya could be used as growth promoters and as well as ethnoveterinary drugs.

CHAPTER SIX
SUMMARY

The current prospective study was conducted on twenty poultry farms (n=20) in Rajshahi Metropolis, Bangladesh from January to December 2013 to assess the sanitation and hygiene quality of small scale commercial poultry farms by structured questionnaire survey through personal interview. The Ranges of disease incidence (DI) was recorded 0.54-13.3%. Among different types of poultry flocks, disease incidence was higher in broiler (mean DI= 6.92%) rather than Cock (3.74%), Sonali (3.18%) and layer (2.89%). Further diseases incidence in poultry correlated with the hygiene and sanitation status of the poultry farms along with attendant personnel. Minimum rate of disease incidence was found in those poultry farms where deep tube-well water (mean DI= 2.4%) were used. In consideration of personal hygiene and sanitation status along with waste management system, only 15% poultry farm sored 'satisfactory' level, 85% scored 'not satisfactory'.

Microbial quality assessment is an integral part of any consumer product development as it gives a clue to the safety and keeping quality of the feed. Total aerobic plate count (APC) of poultry feed samples were recorded as 0.28×10^4 to 2.8×10^8 cfu/g and 33.33-83.33% feed samples showed $>10^6$ cfu/g, because in case of sanitary quality assessment $>10^6$cfu/g considered as hazard point. On the other hand the ranges of cfu of total coliforms were counted asm 0.1×10^4 to 9.6×10^4/gm. The highest cfu was counted 9.6×10^5/gm at Peyarul poultry farm and the lowest was 0.1×10^4/gm at Mostak poultry farm. In case of cfu of *E. coli* were counted as 0.0 to 1.80×10^4/gm. The highest cfu was counted as 1.80×10^4/gm at Juwel poultry farm. *E. coli* was not detected at Roshid and Ahab poultry farms but the ranges of cfu of total Salmonella were counted as 0.0 to 0.85×10^4/gm. The highest cfu was counted 0.85×10^4/gm at Juwel poultry farm and the lowest was counted as 0.0/gm at Peyarul and Mostak poultry farms.

For isolation of enterobacteria, selective media as MacConky, EMB and SS were used. On the basis of morphological characteristics total 44 enterobacterial isolates were isolated. Then the isolates were selected for biochemical test according to

Berger's Manuels and confirmed by Micro Rao online Bacteria identification software. The isolates (SGE-1, MSE-5, ISE-4, JGE-8, JSM-40, PLM-42) were identified as *Escherichia coli* with the probability of 99.91% ; Isolates (MLE-3, MJE-9, IGM-32, JGM-33, BSE-38) were identified as *Klebsiella* sp. with the probability of 100% ; the isolates (RGS-13, JSS-14, SGS-16, MLS-18, AGS-21) were identified as *Salmonella* sp. with the probability of 93.2%; the isolates (ASS-15, MGS-17, PLS-19, RGS-20, RSS-23, MSS-24) were identified as *Shigella* sp. with the probability of 94.12% ; the isolates (JSE-2, MSM-30, PLM-27, KSM-31) were identified as *Enterobacter* sp. with the probability of 99.03% ; the isolates (SSE-6, PLE-7, IGE-10, SGE-11, MGE-12, JGS-22, JSE-36, SGE-39, MGM-41, MSM-44) were identified as *Citrobacter* sp. with the probability of 99.93% and the isolates(RGM-25, AGM-26, MLM-28, RSM-34, AGE-37, MSM-43, ISM-44, RGE-35) were identified as *Proteus* sp. with the probability of 100%.

Total 44 isolates were tested on blood agar medium and only 12 isolates (JSE-2, ISE-4, JGE-8, JSS-14, ASS-15, MGS-17, MLS-18, MSM-30, IGM-32, JGM-33, BSE-38 and JSM-40) showed β-hemolytic activity. Then the isolates were ingested to chicken to justify the virulence capability of the isolates and observed that out of 12 isolates, isolates JSS-20, MGS-17 and IGM-32 showed highly toxic reactivity and the chickens were died after 6 to 9 days. Isolates JSE-2, ASS-15, MLS-18, JGM-35 and JSM-40 showed several sickness symptoms after 6 days while isolates JGE-8, MSM-30 and BSE-38 showed little sickness and isolate ISE-4 showed watery stool symptom. In control, no symptom was found and all the chickens were remained healthy. For detection of pathogenic potentiality of the selected isolates Rose Bengal Agglutination test (RBAT) was performed. From the results it was observed that the six bacteria namely as *Enterobacter* sp. (JSE-2), *E. coli* sp. (ISE-4), *Salmonella* sp. (JSS-14), *Shigella* sp. (MGS-17), *Klebsiella* sp. (IGM-32), *Klebsiella* sp. (BSE-38) showed strong agglutination reactivity in RBAT test which belong in five species. Finally the five pathogenic bacteria (*Enterobacter* sp., *E. coli* sp., *Salmonella* sp., *Shigella* sp., *Klebsiella* sp., and *Klebsiella* sp.) were selected for further experiment.

Identified five entero pahogenic bacteria were tried to control by six plant extracts namely *A. indica*, *C. papya, Z. officinale, C. dactylon, M. arvensis* and *P. pterocarpum.* The yield percentages of the plant extracts were varied and the results showed the highest yield was obtained 15.5% in ethanol extract of *C. dactylon* while the lowest was 7.75% in *A. indica*. In case of methanol extract the highest yield was recorded 13.5% in *C. dactylon* and the lowest was 6.25% in *A. indica*. In case of aqueous extract the highest yield was recorded 10.5% in *P. pterocarpum* and the lowest was 5.0% in *C. papaya.*

From the quantitative estimations it revealed that the extracts contained alkaloid, flavonoid, saponin, tannin and steroid. Besides this, 12 reported antimicrobial phytocompounds as, Oxalic acid, Ascorbic acid, Galic acid, glycine, Cystein, β-sitosterol, Alanine, tryptophan, threonine, vanilic acid, phenilalanine, leusine and citrate were detected by HPLC. HPLC result clearly reveals that the maximum numbers of phytoconstituents (30) are present in *A. indica* extract as compared to other five selected plant extracts as 27 for *C. papaya*, 19 for *P. pterocarpum*, 15 for *Z. officinale,* 14 for *M. arvensis* and the lowest numbers of compounds (12) were found in *C. dactylon.*

The studied plant extracts displayed various degrees of antibacterial activities. The zone of inhibition was varied with the concentration of different plant extracts against the pathogen. The results showed that among the six plant extracts, *C. papaya* was the best as creating larger size of zone of inhibition (14.8±0.44) against *Shigella* sp. The poorest performance occurred by *P. pterocarpum* which scored 7.0±0.58mm against *Klebsiella* sp. The rank of zone of inhibition of the plant extracts were found as *C. papaya* > *A. indica* > *Z. officinale* > *M. arvensis* > *C. dactylon* > *P pterocarpum.*

In comparative analysis on zone of inhibition (mm) values of six plants against selected pathogenic bacteria were investigated to find out the potentiality against five pathogenic bacteria to escape the lethal or antagonistic action. It was found that among the five entero pathogenic bacteria *Shigella* sp. showed strongest

microbial sensitivity. The rank of microbial sensitivity of the pathogenic bacteria was as *Shigella* sp. > *Salmonella* sp. >*E. coli* > *Klebsiella* sp. > Enterobacter sp. Comparative analysis on zone of inhibition (mm) values of three solvents against the pathogenic bacteria was done to detect which solvent played better role. From the comparison it reveals that among the three solvents ethanol played best role and the rank were as Ethanol > Methanol > Aqueous.

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium and the MBC is identified by determining the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculums by ≥99.9%. Considering MIC and MBC value, *A. indica* showed the best performance against all identified pathogenic bacteria. Comparative analysis on average MIC and MBC values, the potentiality to inhibit entero pathogenic bacteria of six plant was found as *A. indica* > *C. papaya* > *Z. officinale* > *M. arvensis* > *C. dactylon* > *P. pterocarpum*

In *in vivo* trial *A. indica* leaves and papaya fruit extracts were used to control enterobacteria associated diseases of poultry. From the results it was observed that the weight of the poultry was rapidly increased in TN_1 (Additive), TN_2 (Neem) and TN3 (Additive+Neem) treatments compare to others. At 45^{th} day, the maximum weight gain was observed in TN_3 (Add.+Neem) which were 11.16% higher than control. For C. *papaya* it was observed that the weight of the poultry was rapidly increased in TP_1 (Additive), TP_2 (Papaya) and TP3 (Additive+Papaya) compare to others. At 45^{th} day, the maximum weight gain was observed in TP_3 (Additive +Papaya) which were 11.37% higher than control. So, these two plants could be used in EVM to control enterobacteria induced diseases in poultry.

CHAPTER SEVEN
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