COMPARATIVE EFFICACY OF SYNTHETIC ANTIFUNGALS AND SELECTIVE MEDICINAL PLANTS AGAINST ASPERGILLOSIS IN BROILERS

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Roll No. 0213/01

Registration No. 176

Session: 2013-2014

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Pharmacology

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DECEMBER 2014

╣ DEDICATION ╠

To my Mother, thank you for your constant empowerment. You are the most positive person I know

&

To my Father, my mentor. I am forever grateful that you took me straight to the top and made me skip the bunny slope

Authorization

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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DECEMBER 2014

Acknowledgements

I would like to utter my deepest sense to "The Almighty Allah", who enables me to complete the research work and dissertation successfully for the Degree of Master of Science (MS) in Pharmacology under the Department of Physiology, Biochemistry and Pharmacology (DPBP), Chittagong Veterinary and Animal Sciences University (CVASU).

I would like to expresses the first and foremost heartiest appreciation, deepest sense of gratitude and best regards to my learned supervisor Prof Dr. A. K. M. Saifuddin. It was my immense pleasure and amazing experience to work under his constructive and effective supervision during the entire period of research work. Without his guidance it would not be possible for me to complete the research.

I feel pleasure to convey my profound appreciations to my co-supervisor, DR. Mohammad Mahmudul Hassan, Associate Professor, DPBP, CVASU for his scholastic guidance, suggestions and inspiration during write up the dissertation successfully. I would like to give special thanks to DR. Amir Hossan Shaikat, Assistant Professor, DPBP, CVASU for his precious advice, constructive criticism and huge cooperation during experimental works.

I am also thankfull to DR. Md. Zohorul Islam, DR. Babu Kanti Nath, DR. Md. Saiful Bari and DR. Md. Shafiqul Islam, CVASU for their cordial help during the experimental wok.

I like to acknowledge the support, cooperation and encouragement received during my research from other teaching, technical and non-technical staffs of DPBP. I sincerely thank to the directorate of research and extension, CVASU for giving me a research grant to accomplish my research work.

I am immeasurably grateful to my friends (Nahid, Mahbub, Zaman, Momin, Shohel, Imran, Noman, Johir, Al-Amin, Mukti, Sumona, Ayesha, Salma), elder brother (Yusuf) and juniors (Fakhrul, Rostum, Farhana, Sankar, Sabrina, Mizan, Tofazzal) for their enormous assist, mental support and encouragement during the MS research work.

Last, but not the least, I am ever indebted to my beloved parents and younger brother for their immense sacrifice, blessing and encouragement.

The Author
December, 2014

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Acronyms Used

ANOVA	Analysis of Variance
%	Percentage
ESR	Erythrocyte Sedimentation Rate
PCV	Packed Cell Volume
Hb	Hemoglobin
DLC	Differential Leucocytes Counts
TG	Triglycerides
HDL	High Density Lipo-protein
LDL	Low Density Lipo-protein
CHOL	Cholesterol
ALT/ SGOT	Alanine Amino-Transferase
AST/ SGPT	Aspartate Amino-Transferase
TP	Total Protein
Alb	Albumin
SDA	Saboured Dextrose Agar
PDA	Potato Dextrose Agar
AFB_1	Aflatoxin B_1
TLC	Thin Layer Chromatography
ME	Metabolizable Energy
CP	Crude Protein
FCR	Feed Conversion Ratio
BCRDV	Baby Chick Ranikhet Disease Vaccine
DLS	District Livestock Station
LRI	Livestock Research Institution
IUCN	International Union for Conservation of Nature

Comparative Efficacy of Synthetic Antifungals and Selective Medicinal Plants against Aspergillosis in Broilers

Summary

An experimental study was conducted on broiler birds for evaluation of efficacy on synthetic antifungals (Nystatin Sulphate, 0.1% $CuSO_4$) and medicinal plants (*Azadirachta indica*, *Ocimum tenuiflorum*, *Allium cepa*, *Allium sativum*) against aspergillosis during July to December, 2014. This efficacy was determined on the basis of growth performance, hematological and biochemical alterations in birds. A total of 150 Cobb-500 day-old chicks were divided into seven subgroups as Control (T_0), Onion (T_1), Garlic (T_2), Neem (T_3), Tulsi (T_4), 0.1% $CuSO_4$ (T_5) and Nystatin sulphate (T_6) comprising 21 birds in each. *Aspergillus sp.* was challenged through feed and water to all subgroups except T_0 at Day-16 and Day-20. Blood samples were collected three times (infection exposure, during treatment and post treatment) from three randomly selected birds of each subgroup and subsequent hematology and biochemical analysis were done. Feed intake data with live weight was recorded in every seven days interval to assess growth performances. Garlic showed better antifungal efficacy with highest live weight (1354g ± 76) and improved feed conversion (1.66) compared to control followed by Onion, Nystatin Sulphate and Neem. Tulsi also had antifungal efficacy but growth performance (Live Weight 1166g ± 82, FCR 1.90) was inconsistent. Significant variation ($p \leq 0.05$) was observed in Hemoglobin (Hb), Total Erythrocyte Count (TEC), heterophil, lymphocyte, monocyte, Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) due to infection. Triglyceride (TG), Low Density Lipoprotein (LDL), albumin and Erythrocyte Sedimentation Rate (ESR) were significantly raised after treatment. Cholesterol and High Density Lipoprotein (HDL) were varied in Neem followed by control and reduction of glucose was found in Garlic and Nystatin groups. However, significant alteration was present on AST and ALT in both Garlic and Neem groups and increased albumin and LDL were frequent only on Neem. TEC was significant in Nystatin, Onion and Control and Hb was decreased in 0.1% $CuSO_4$ group. On the other hand, lymphocyte elevation with reduced heterophil was observed both in Neem and Nystatin groups and simultaneously monocyte was decreased in Control, Onion and 0.1% $CuSO_4$ groups. It is concluded that, Garlic possesses a good antifungal efficacy with an improved growth performance than other medicinal plants and synthetic antifungals.

Key Words: *Aspergillus*, Broilers, Synthetic antifungals, Medicinal plants, Growth performances, Hematology, Serum biochemistry.

Chapter-1: Introduction

Fungal infections are common in all kinds of poultry but less prevalent compared to bacterial and viral infections (Dahlhausen, 2006). Aspergillosis, one of the most common pathogenic fungal diseases has economic impact on poultry industries (Singh et al., 2012) either due to their direct infectious nature (Dahlhausen, 2006) or production of mycotoxins on poultry feed (Dhama et al., 2013; 2007; Jand and Singh, 1995). A worldwide distributed filamentous saprophytic fungus, *Aspergillus fumigatus* is considered as a major respiratory pathogen in almost all domesticated and wild avian species to cause aspergillosis (Arné et al., 2011; Tell, 2005). Lungs and airsacs are usually involved; trachea, syrinx and bronchi are also affected. Clinical signs include dyspnoea, gasping, hyperpnoea with panting, nonproductive coughing, wheezing, cyanosis and sometimes nasal discharge (Dhama et al., 2011; Ganguly et al., 2011; Atasever and Gumussoy, 2004). Chronic form is more likely to occur in older birds that have been in captivity (Arné et al., 2011; Jordan and Pattison, 1996).

Antifungal drugs have been used for the treatment of aspergillosis in several poultry species. Many studies have been conducted to investigate the efficacy of various drugs, such as Amphotericin-B against *Aspergillus spp* (Galiger et al., 2013; Bonar and Lewandowski, 2004; Arikan et al., 1999). Another therapeutic intervention against *Aspergillus* is the use of voriconazole, clotrimazole, miconazole, ketoconazole, itraconazole, fluconazole, 5-fluorocytosine and nystatin sulphate (Dhama et al., 2012; Beernaert et al., 2009a; Beernaert et al., 2009b; Burhenne et al., 2008; Scope et al., 2007; Pericard, 2005). Nystatin sulphate has been used for the management of mucocutaneous candidiasis now-a-days which is also active against *Aspergillus*, *Histoplasma* and *Coccidioides spp*. to a lesser extent (Wallace and Lopez-Berestein, 1998). Treating litter with Nystatin sulphate and 0.1% $CuSO_4$ can reduce fungal content while 0.1% $CuSO_4$ alone as treatment has been shown fungicidal activity against *Aspergillus spp.* (Johnson and Fiske, 1935).

On the other hand, plants are the natural reservoir of many antimicrobial agents. There are many plant originated products that have been made into medicines and

used in treatment of bacterial, viral and fungal diseases (Bernal et al., 2011). The use of plants is increasing day by day as they have minor or no side effects (Jordan et al., 2010; Tapsell et al., 2006). In Bangladesh, different parts of medicinal plants have been used to cure specific ailments. Bangladesh is endowed with vast resources of medicinal plants such as Neem (*Azadirachta indica* A. Juss.), Tulsi (*Ocimum sanctum*), Sajna (*Moringa oleifera*), Pineapple (*Ananas comosus*), Custard apple (*Annona reticulata*), Onion (*Allium cepa*), Garlic (*Allium sativum*), Turmeric (*Curcuma longa*), Zinger (*Zingiber officinale*), Betel (*Piper betle*), Shatamuli (*Asparagus racemosus*), Sarpagandha (*Rauvolfia serpentian benth*), Ghritkumari (*Aloe indica*), Lajjabati (*Mimosa pudica*), Ulatkambal (*Abroma augsta*), Dadmardan (*Cassia alata*), Durba grass (*Cynodon dactylon*) etc. are using as anthelmintic, antimicrobial, antifungal, astringent and hemostatics (Akhtar et al., 2000).

Neem (*Azadirachta indica* A. Juss.) is a tropical evergreen tree native to Indo-Bangladesh sub-continent (Girish and Bhat, 2008) and is the most promising tree of the 21st century. Most of the parts of this plant such as leaves, seeds, bark and roots contain compounds that have antiseptic, anthelmintic, antibacterial, antiviral, antipyretic, anti-inflammatory, antiulcer and antifungal uses (Anonymous, 2006; Sateesh, 1998). It is a known inhibitor of fungal growth and high antimycotic activity (Bansod and Rai, 2008 and Jacobson, 1986). Crude aqueous extracts were found to have an inhibitory effect on the growth of *A. flavus* and *A. parasiticus* (Thanaboripat et al., 2000).

Tulsi (*Ocimum sanctum*) is known as "the elixir of life" since it promotes longevity. Various parts of this plant are used in ayurveda and siddha systems of medicine for prevention and cure of many illnesses such as antitussive, antihistaminic, antifungal, common cold, cough, flu, influenza, skin diseases and wound etc. Extract of Tulsi effectively worked against *Aspergillus flavus* and aflatoxin B_1 (Reddy et al., 2009).

Both bulb of *Alliums spp.* (Onion-*Allium cepa* and Garlic-*Allium sativum*) have antifungal activity. Onion is considered to have anthelmintic, antioxidant, antifungal, hemostatic, antiseptic, carminative, diuretic, expectorant and febrifuge activities. In traditional medicine, Onion is used for colds, coughs, flu and bronchitis (Kumar et al., 2010). Onion oil is effective to gradually reduce fungal growth and aflatoxin

production by *Aspergillus flavus* and *A. parasiticus* var. *globosus*. Fungal growth and production of sterigmatocystin and rubratoxin A by *Aspergillus versicolor* were completely inhibited by the addition of Onion oil (Zohri et al., 1995). On the other hand, Garlic has antibiotic, antibacterial and antifungal action (Ismaiel et al., 2012). It is a fungistatic substance, has proved itself against *Aspergillus* (An et al., 2009; Ogita et al., 2009; Tedeschi et al., 2007).

In recent years, traditional medicinal plants are used as an alternative to synthetic antifungal drugs. However, very limited informations are available about the activities of traditional plants as antifungal against aspergillosis in broilers. Therefore, this experiment was designed to study *in vivo* antifungal activity of Nystatin Sulphate and 0.1% $CuSO_4$ with ethanol extracted Neem, Tulsi, Onion and Garlic against *Aspergillus spp.*

1.1 Specific objectives:

- To investigate the comparative antifungal efficacy between synthetic antifungal and ethanol extracted medicinal plants
- To know the effects of synthetic antifungals and medicinal plants on Feed Conversion Ratio (FCR) and blood parameters
- To evaluate the effects of synthetic antifungals and medicinal plants on serum biochemistry

Chapter-2: Review of Literature

2.1 Aspergillosis

Aspergillosis, commonly known as **brooder's pneumonia**, is caused by *Aspergillus fumigatus*, most pathogenic fungi affecting poultry (Arne et al., 2011). But *A. flavus* has also been the culprit associated with many cases, respiratory infection by *Aspergillus spp.* has been reported in almost all types of poultry birds viz., layer cockerels (Steinlage et al., 2003), broilers (Martin et al., 2007), growers (Zafra et al., 2008) and turkey poults (Olias et al., 2010).

2.1.1 Epidemiology with pathogenesis

Aspergillus fumigatus infection occurs more frequently in poultry as the spores are smaller than other *Aspergillus spp.* (Arne et al., 2011) such as *A. terreus, A. glaucus, A. nidulans* and *A. niger* (Dhama et al., 2012; Beernaert et al., 2010; Joseph, 2000). Mainly spores of the fungi are resistant in nature. Poultry birds coming in contact with the spores through contaminated feed or dry litter, gets affected after inhaling the spores (Cacciuttolo et al., 2009; Nardoni et al., 2006). The predisposing factors for spore generation and dissemination in the air/environment include warm environment, humidity, poor ventilation and sanitation along with long term storage of feed (Khosravi et al., 2008; Tell, 2005; Phalen, 2000). Besides direct extension of the infection through air sac wall, disseminated mycosis also occurs by haematogenous spread. Hyphae, which are known as tissue, are angio-invasive (Dahlhausen et al., 2004) and as well as host cells play a role in this spreading mechanism.

The disease develops in brooder stages in chicks as well as passerine birds, especially below 3 days of age (Chauhan and Roy, 2008). High humidity and moderate temperature contributes significantly towards the occurrence and spread of aspergillosis (Dhama et al., 2008). Contaminant like lead acts as a precipitating factor, especially in geese (Kapetanov et al., 2011).

Aspergillosis primarily causes high morbidity and mortality especially in young chicks (Arne et al., 2011; Redig, 2005). Acute aspergillosis occurs as a result of inhaling high number of spores, wherein severe disease outbreaks in young birds are

characteristically observed. Morbidity and mortality are high (30-90%) in it and can be seen within 24-48h of infection. Chronic form occurs sporadically and generally observed in adult breeder birds (particularly turkeys). Proteases and toxic secondary metabolites secreted by the fungus contribute to virulence (Pena et al., 2010; Tekaia and Latge, 2005) along with gliotoxin (20-70μg/g in poultry diet), a highly immunosuppressive mycotoxin (Vanderheyden, 1993). However, lytic changes are observed in the epithelium of the upper airway due to inhalation of conidia by short duration of exposure (Nganpiep and Maina, 2002).

2.1.2 Clinical signs

Affected birds show gasping along with respiratory rattle and milder form of anemia within 8-10days in birds' upto 2 weeks of age (Dhama et al., 2011; Atasever and Gumussoy, 2004). Yellow colored pin point lesions are visible in lungs, air sacs and various organs miliary to larger granulomatous foci (Ganguly et al., 2011). Sometimes small yellow green fungus found in all body cavities with dry consistency of lungs, thickened air sac and suppurate filled bronchioles. Cutaneous aspergillosis with necrotic granulomatous dermatitis was observed in chicken and pigeons (Beernaert et al., 2010; Cacciuttolo et al., 2009; Nardoni et al., 2006).

Survivors often develop chronic disease due to pulmonary insufficiency or neurological disorders as lethargic and stunted growth (Beernaert et al., 2010; Calderone and Fonzi, 2001). Rapid death due to aspergillosis can flare up avian influenza even though there is no connection between the two diseases and requires laboratory attention to distinguish them (Kradin and Mark, 2008).

2.1.3 Diagnosis

Individual test does not provide reliable diagnosis and therefore confirmatory diagnosis requires disease history, clinical signs, hematological and biochemical profile, serology, radiographic changes along with endoscopy and cultural examination of the fungus (Jones and Orosz, 2000). The clinical signs depend on the form of the disease and involvement of organ (Jones and Orosz, 2000), thereby requiring the disease to be differentiated from other systemic diseases of respiratory tract (Jones and Orosz, 2000).

On necropsy, varying degree of miliary granulomatous foci was noted (Vanderheyden, 1993). Definitive diagnosis requires demonstration of the organisms by cytology or histopathology and subsequent identification by culture (Dahlhausen et al., 2004). Isolation of the fungus alone does not confirm the infection status because *Aspergillus* organisms are ubiquitous contaminants (Flammer and Orosz, 2008; Jensen et al., 1997). However, plentiful culturing from any organ should be considered for diagnosis (Redig, 2005; Jensen et al., 1997). Heart along with organs of respiratory system like larynx, trachea and lungs are important for histopathological examination. Microscopic lesions are suggestive but not helpful in species identification because *in vivo* hyphae of hyaline filamentous fungi are very similar and their *in situ* manifestations are not pathognomonic (Cray et al., 2009a; Tekaia and Latge, 2005; Kaufman et al., 1997). Thus, immunohistochemistry usually can provide confirmatory diagnosis where few reports are demonstrated using monoclonal or polyclonal antibodies for diagnosing aspergillosis in birds (Beytut, 2007; Beytut et al., 2004; Jensen et al., 1997).

2.1.4 Treatments, prevention and control

Table 1: Administration routes and doses of some antifungals against avian aspergillosis

Antifungal agent	Administration route	Dose
Amphotericin B	Intravenous Intrathecal Nebulization Into air sac Topical (Wound)	1.5mg/kg every 8h interval 3-5 days (Joseph et al., 1994), 10-14 days (Jenkins, 1991)
		1.35mg/kg 24h interval (liposomally encapsulated amphotericin B) (Bonar and Lewandowski, 2004)
		1mg/kg 24h interval 10-14 days (Jenkins, 1991)
		1mg/ml 15min 5-7 days every other week (Orosz and Frazier, 1995) 12h interval (Joseph et al., 1994)
		Dose not specified (Flammer, 1993)
Nystatin Sulphate	Oral	20000IU/ml every 12h interval 4 days
Clotrimazole	Topical Nebulization	Dose not specified (Flammer, 1993)
		10mg/ml polyethylene glycol for 30-45min 24h interval 3 days on/2 days off (1-4 months) (Orosz and Frazier, 1995; Joseph et al., 1994)
Enilconazole	Topical Nebulization Disinfection	Dose not specified (Flammer, 1993)
		0.1ml/kg for 30 min interval 24h 5 days on/2days off (raptors) (Heatly et al., 2007)

		Environment: flush with solutions as recommended for use in poultry houses (Flammer, 1993)
Fluconazole	Oral, Intravenous	5mg/kg 24h interval 7 days (Flammer, 1993)
		15mg/kg 12h interval (psittacines) (Pericard, 2005)
Itraconazole	Oral	5-15mg/kg 12h interval with food for 7-21 days (Bauck et al., 1992)
		10mg/kg 24h interval 3 weeks (Verstappen and Dorrestein, 2005) (falcons) (Jones et al., 2000)
		15mg/kg per orally 12/24h interval (Abrams et al., 2001), 10-20mg/kg 12/24h interval (Arikan and Rex, 2000; Flammer, 1993)
		5mg/kg 24h interval 30 days (African grey parrots) (Orosz and Frazier, 1995)
		5-10mg/kg 24h interval (Amazon parrots) (Orosz et al., 1996), 6mg/kg 12h interval (pigeons) (Lumeij et al., 1995)
		Preventive: 10mg/kg 24h interval 10 days (Forbes, 1992), 20mg/kg 24h interval (Meredith, 1997), 15-25mg/kg/day for 1 week (Xavier, 2008)
Ketoconazole	Oral	10-30mg/kg 12h interval 21 days (re-suspending in orange juice 5 days interval) (Bauck et al., 1992)
		20-30mg/kg 12h interval (Flammer, 1993), 30mg/kg 12h interval 14-30 days (Orosz and Frazier, 1995)
Miconazole	Topical Intrathecal Nebulization	12h interval (Suedmeyer et al., 2002; Abrams et al., 2001; Orosz and Frazier, 1995)
		pH balanced solution, aqueous base, dilute in saline: 15-20min 12h interval (Arikan and Rex, 2000; Orosz and Frazier, 1995)
		5mg/kg, 10mg/ml, 12h interval (diluted to maximum 0.5ml with saline) (Westerhof, 1995)
Terbinafine	Oral Nebulization	10mg/kg 12-24h interval, 15mg/kg 12h interval (psittacines) (Flammer and Orosz, 2008)
		Combined with itraconazole (Flammer, 2006).
Voriconazole	Oral	10mg/kg 12h interval (pigeon) (Beernaert et al., 2009b), 24h interval (chickens) (Burhenne et al., 2008) (African grey parrots) (Scope et al., 2007)
		12-18mg/kg 12h interval (African grey parrots) (Flammer and Orosz, 2008)
		12.5mg/kg 12h interval, 3 days loading dose, then 24h interval (raptors) (Di Somma et al., 2007; Schmidt et al., 2007).
5-fluorocytosine	Oral	50-100mg/kg 12h interval (Flammer, 1993)
		60-250mg/kg 12h interval (cage birds); 40mg/kg 6-8h interval (raptors) (Jenkins, 1991)
		150-250mg/kg for 21 days (Bauck et al., 1992)

120mg/kg 12h interval 3 weeks (Westerhof, 1995)
120mg/kg 6h interval (Joseph et al., 1994)

2.2 Nystatin sulphate

Nystatin, the first polyene drug to be identified, was originally extracted from *Streptomyces nousei*. It is similar in structure to amphotericin B and acts by binding to ergosterol, a component of the fungal cell membrane. Alteration in membrane permeability allows the release of potassium, sugars and metabolites, resulting in fungal death (Wallace and Lopez-Berestein, 1998; Hammond, 1977). Nystatin is active against a wide variety of fungal pathogens including *Candida, Aspergillus, Histoplasma* and *Coccidioides spp*.

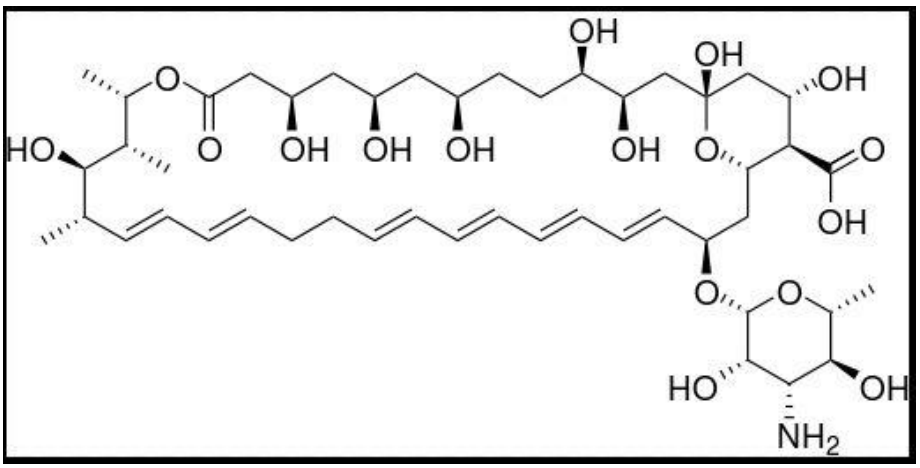


Figure 1: Chemical composition of Nystatin sulphate (Wikipedia, 2015)

Aspergillosis in chickens and turkeys are very often associated with contaminated litter. Treating litter with Nystatin and $CuSO_4$ can reduce mold content (Dyar et al., 1984). This drug is not very much useful for aspergillosis. The drug given in feed or by injection, had good results in water fowl in captivity (O'Meara and Witter, 1971).

2.3 Copper sulphate

Copper Sulphate is a widely used chemical compound comprised of copper, sulpher and oxygen whose chemical formula is $CuSO_4$. Crystal of $CuSO_4$ is often bright blue and the substance was known as Blue Vitriol or Bluestone.

A concentration of 0.1% $CuSO_4$ in the diet of bacon, pigs and broiler chickens stimulate appetite and increase growth rate with a marked improvement in feed

conversion. This compound has been shown as fungicidal to *Aspergillus spp.* (Johnson and Fiske, 1935) in poultry during summer periods at concentrations above 0.4μg/ml. On the other hands, $CuSO_4$ at 60g/quintal of feed for 6 days is effective for treatment of aspergillosis. In outbreaks, drinking water with 1:2000 aqueous solution of $CuSO_4$ needs to be provided. Normally, copper plays a role in reducing cholesterol in plasma of poultry. It also lowers fat content, increases meat protein and feed efficiency.

Significant reductions in cholesterol and triglyceride and an elevated concentration of HDL were observed in chickens fed with 250mg $CuSO_4$ in per kg feed at 3^{rd} and 6^{th} week age of birds. Total cholesterol in meat decreased significantly in the birds. Growth performance was measured in terms of live weight gain, cumulative feed intake and FCR at the end of Day-21 and Day-42 of the experiment and result was found as beneficial for the chickens (Samanta et al., 2011).

Comparative influence of organic and inorganic dietary copper on growth, blood characteristics were evaluated in Arbor-Acre unsexed broilers (Jegede et al., 2011). A total of 480 broilers were fed on diets containing $CuSO_4$ or copper proteinate at concentrations of 50, 100 or 150mg/kg as copper supplementation. At Day-28, copper proteinate birds had improved FCR compared with $CuSO_4$. Feed consumptions for the two copper sources were not significantly differed. Copper proteinate supplementation was more effective in promoting growth, increased hemoglobin concentration but reduced triglyceride and cholesterol.

2.4 Medical herb

Herbs are plants of which the leaves, stems, flowers, seeds and roots are used for flavoring, dishes or for medicinal purposes (Hashemy and Davoodi, 2011; Sandhu and Heinrich, 2005). Herbalism or herbal medicine is used of whole plants or its different parts of plants for medicinal purposes as conventional medicine. Modern medicine recognizes herbalism as a form of alternative medicine (Kala, 2006).

2.4.1 The history of herbalism

As far as records go, it appears that the king Hammurabi of Babylon (1800 B.C.) prescribed the use of herbals (Petrovska, 2012). Approximately 5000 years ago, the

root of Chinese medicine is based largely on herbalism. The Chinese emperor Chi'en Nung put together medicinal plants book called 'Pen T'sao'. It contained over 300 herbs including Chinese EPHEDRA, which is still widely used (Wiart, 2006). The medicinal use of plants in the Indian sub-continent was found in the Rig Veda (4500-1600 BC). 'Charaka Samhita' the comprehensive Indian herbal, cites more than 500 medicinal plants (Sumner, 2000). But, the first written record of herbs used as medicine was makeover 5000 years ago by the Sumerians, in ancient Mesopotamia. Egyptian hieroglyphs also hint about the use of herbs (Barry and Baek, 2009).

Then it was the history of developing pharmacopeia. The Greek physician, Hippocrates (460-370 BC), compiled the use of 300-400 medicinal plants in 'Materia Medica'. The work of Dioscorides 'De Materia Medica' was the predecessor of all modern pharmacopoeias and an authoritative text for 600 medicinal plants, 35 animal products, and ninety minerals. 'De Materia Medica' remained the authoritative reference of herbalism into the 17^{th} century (Kala and Sajwan, 2007). In 1653, Nicholus Culpepper wrote a physical directory and a few years later produced 'The English Physician'. This respected herbal pharmacopeia was one of the first manuals could use for health care and still widely referred and quoted. The first U.S. pharmacopeia was published in 1820. It was periodically revised and became the legal standard for medical compounds in 1906. But, the development of extracting and synthesizing the active ingredients from plants for drug production outcastes the herbal treatments (Hopking, 2004; Morgan, 2002).

2.4.2 Medicinal plants of Bangladesh

In Bangladesh 5000 species of angiosperm are reported to occur (IUCN, 2003). The number of medicinal plants included in the 'Materia medica' of traditional medicine in this subcontinent about 2000 at present stands. More than 500 of such medicinal plants have so far been enlisted as growing in Bangladesh (Ghani, 2003; Saha et al., 2006). About 58 different medicinal plants are being used in various lesion, itching, dysentery, skin disease, cold/cough, headache, fever, appetizer etc in rural Bangladesh (Hossain, 2003). The knowledge of using herbal medicine was disseminated by local kabiraj (Amin et al., 2008).

2.4.3 Effect of medicinal plants on *Aspergillus* infection

Various south-east asian medicinal plants such as Asiatic Pennywort (*Centella asiatica*), Betel Nut (*Areca catechu*), Betel Vine (*Piper betle*), Bitter Cucumber (*Momordica charantia*), Chaa Phluu (*Piper sarmentosum*), Chinese Radish (*Raphanus sativus*), Clove (*Syzygium aromaticum*), Eucalyptus (*Eucalyptus globules*), False Coriander (*Eryngium foetidum*), Hedge Flower (*Lantana camara*), Indian Mulberry (*Morinda citrifolia*), Madagascar Periwinkle (*Catharanthus roseus*), Aloe (*Aloe vera*), Mangosteen (*Garcinia mangostana*), Mandarin (*Citrus reticulate*), Onion (*Allium cepa*), Ginger (*Zingiber officinale*), Pepper (*Piper nigrum*), Pomegranate (*Punica granatum*), Roselle (*Hibiscus sabdariffa*), Non-taai Yaak (*Stemona tuberosa*), Tomato (*Lycopersicon esculentum*), Raang Chuet (*Thunbergia laurifolia*), Turmeric (*Curcuma longa*), Peppermint (*Mentha piperita*), Basil (*Ocimum basillicum*), Thyme (*Thymus vulgaris*) and Water Primrose (*Jussiaeda repens*) were tested to control *A. flavus* (Thanaboripat et al., 2006; 2004). Crude ethanolic extracts of some medicinal plants inhibited fungal growth to various degrees. Extracts from Garlic bulbs, green Garlic and green Onions showed an inhibitory effect against *A. niger* and *A. flavus* (Yin and Cheng, 1998). However, green Garlic and green Onion lost their antifungal activity against *A. niger* after being heated at 80°C and 60°C, respectively.

2.5 *Allium cepa* L. (Onion)

Commonly known as bulb Onion. It contains numerous sulfur compounds, including thiosulfinates and thiosulfonates; cepaenes; S-oxides; S, S-dioxides; mono-, di- and tri-sulfides and sulfoxides (Wiczkowski et al., 2008; Arnault and Auger, 2006; Lanzotti, 2006; Rose et al., 2005).

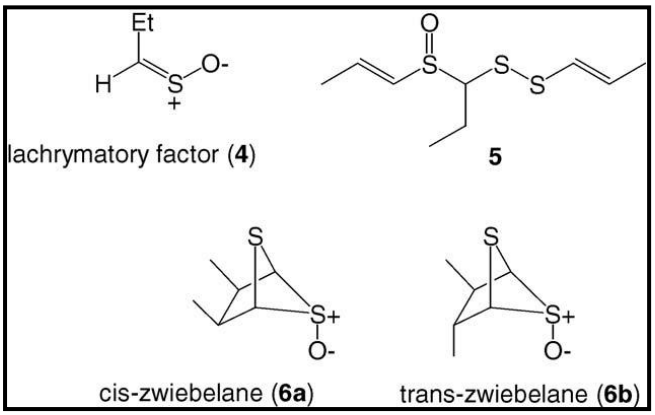


Figure 2: Volatile organosulpher compounds of Onion (Lanzotti, 2006)

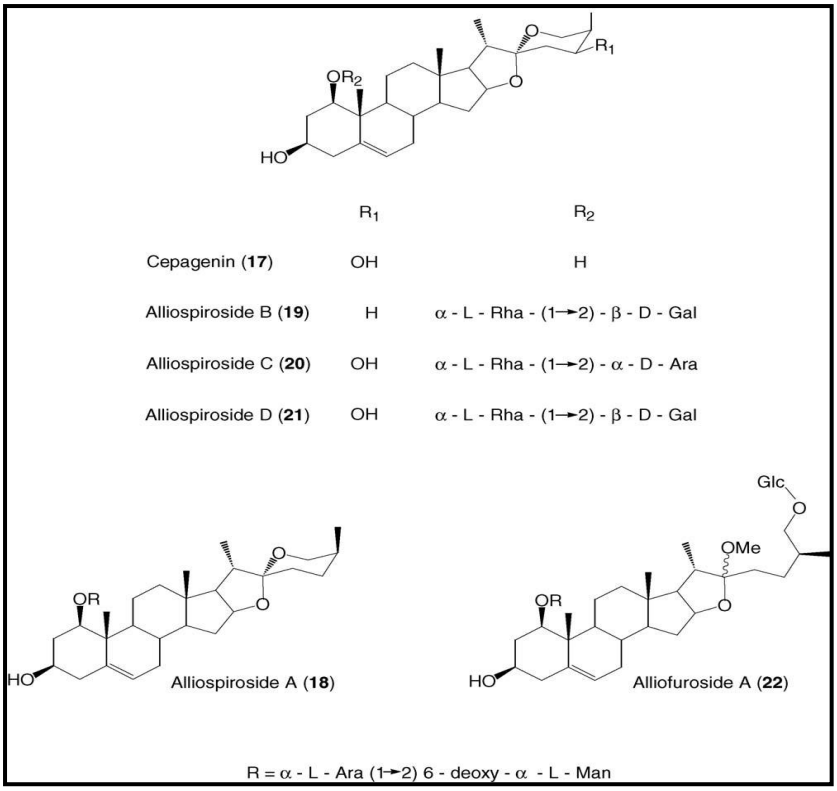


Figure 3: Sapogenin and saponins from Onion (Lanzotti, 2006)

2.5.1 Pharmacological uses of onion

It is considered to have anthelmintic, antioxidant, antiseptic carminative, diuretic, expectorant, febrifuge and vulnerary properties. In traditional medicine, Onion had been used for colds, coughs, flu, bronchitis and heart disease. Fresh Onion can kill germs in mouth and soothe toothache. Recent studies are showing its beneficial effect in the treatment of high blood pressure and high blood cholesterol. It can be a good prevention against cardiovascular disease and even certain head and neck tumors. Some studies suggest that high consumption of Onion, along with Garlic lowers the possibility of stomach cancer for 40%. It can also be used as prevention against osteoporosis and in treatment of blisters, boils and topical scars (Kumar et al., 2010).

2.5.2 Antifungal activity

Onion oil at different concentrations (100, 200 and 500ppm) tested gradually reduced fungal growth and aflatoxin production by *Aspergillus flavus* and *A. parasiticus* var. *globosus*. Fungal growth and production of sterigmatocystin and rubratoxin A by *Aspergillus versicolor* were completely inhibited by the addition of 200ppm Onion oil

(Zohri et al., 1995). Allicepin, a novel antifungal peptide (Wang and Ng, 2004) exerted an inhibitory activity on mycelial growth.

2.5.3 Effect of Onion on biochemical parameters

The lipid lowering action of S-methyl cysteine sulfoxide (SMCS) isolated from *Allium cepa* was investigated in Sprague-Dawley rats fed on 1% cholesterol diet (Kumari and Augusti, 2007). Administration of SMCS at a dose of 200 mg/kg body weight for 45 days ameliorated the hyperlipidemic condition. The lipid profile in serum and tissues showed that concentrations of cholesterol, triglyceride and phospholipids were significantly reduced compared to their untreated counterparts. The total lipoprotein lipase activity in the adipose tissue was decreased with also a decrease in the free fatty acid levels in serum and tissues (Kumari and Augusti, 2007).

The effect of cycloalliin (present at lower levels in fresh Onions) was investigated on lipid metabolism in Sprague-Dawley rats (Yanagita et al., 2003). Body weight and the levels of glucose, plasma triglycerides (TG), plasma total cholesterol (CHOL), plasma high-density lipoprotein cholesterol (HDL), and liver glycogen (LG) were considered (Kook et al., 2009) where those parameters were significantly differed between the untreated and treated hypoglycemic group in Onion supplement studies.

The effect of Onion extract (1ml of Onion juice/100g body weight orally administered daily) was studied (El-Demerdash et al., 2005) to diabetic rats for four weeks. Treatment of the diabetic rats with repeated doses of Onion extract could restore the changes of glucose, urea, creatinine and bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase, alkaline and acid phosphatases activities in plasma, as well as glutathione S-transferase in plasma and liver to their normal levels.

2.6 *Allium sativum* (Garlic)

Common named as Garlic. When crushed, *Allium sativum* yields allicin, an antibiotic and antifungal compound (phytoncide) discovered by Chester J. Cavallito and colleagues in 1944. Fresh or crushed Garlic also affords the sulpher containing compounds allin, ajoene, diallyl polysulfides, vinyldithiins, S-allylcysteine, and enzymes, B vitamins, proteins, minerals, saponins, flavonoids and

maillard reaction products, which are not sulfur-containing compounds. Furthermore, a phytoalexin (allixin) was found, a nonsulfur compound with a γ-pyrone skeleton structure with antioxidant effects (Kodera et al., 1989), antimicrobial effects, antitumor promoting effects, inhibition of aflatoxin B_2 DNA binding (Yamasaki et al., 1991) and neurotrophic effects.

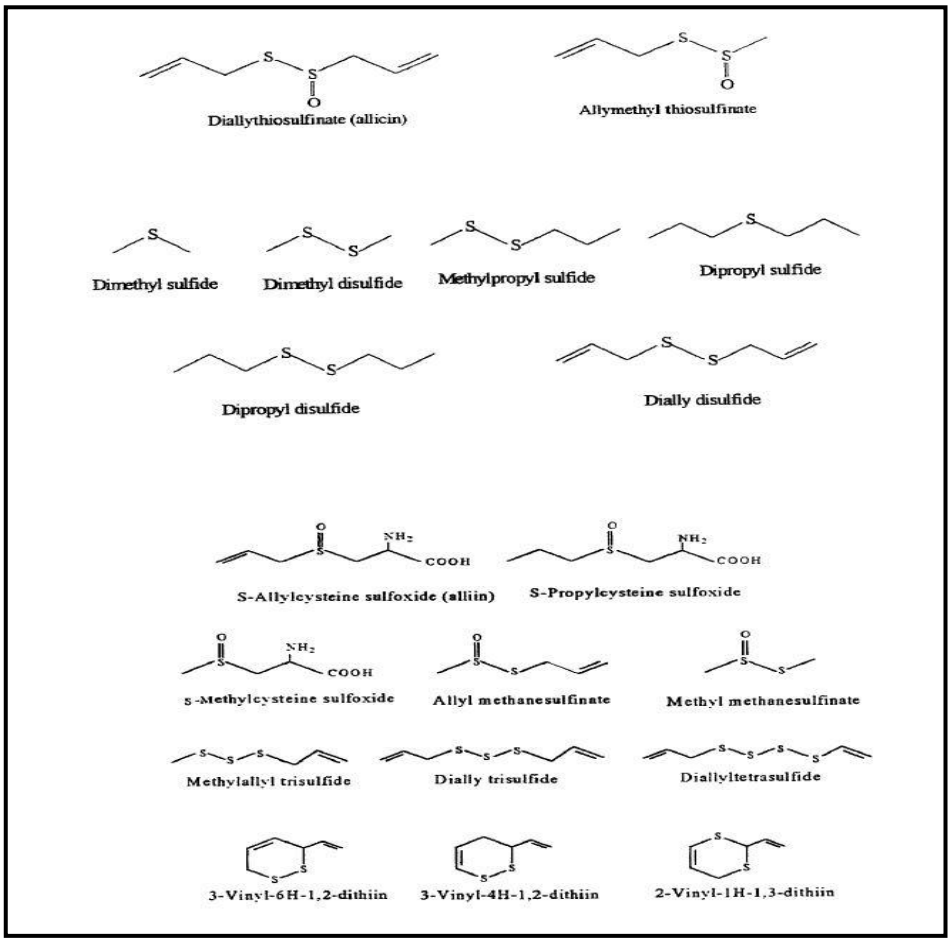


Figure 4: Sulpher compounds from garlic and onion (Ali et al., 2000)

2.6.1 Antifungal activity

Garlic, which consists of allicin being a fungistatic substance, has proved itself against microorganisms, such as *Candida*, *Aspergillus* and *Cryptococci* as an effective anti-fungal substance. Allicin, an allyl sulphur compound from Garlic (An et al., 2009; Ogita et al., 2009), has shown to significantly enhance the effect of Amphotericin B against *Candida albicans* and *Aspergillus fumigatus in vitro* and *in vivo*, although allicin did not exert a fungicidal effect. Antibacterial and antifungal effects of Garlic extracts are sourced from the existence of dialkyl disulfide and allicin (Yoshida et al., 1998; Agrawal, 1996). It has been pointed out that antifungal

effect of garlic extract on *Aspergillus niger*, *A. flavus* and *A. fumigatus* is not available but with acetic acid addition into this extract, in the situation of increasing acidity, fungal development decreases in important ratio (Yin and Tsao, 1999).

2.6.2 Effect of Garlic on chicken's performance

Garlic showed positive effects on the performance of different species. Essential oils of Garlic acts as a digestibility enhancer, balancing gut microbial ecosystem and stimulating secretion of endogenous digestive enzymes and thus improving growth performance in poultry (Cross et al., 2007; Lovkova et al., 2001; Williams and Losa, 2001).

Increase in growth, feed conversion and meat quality were obtained when broilers were fed diets containing 1% or 2% Garlic (Bampidis et al., 2005; Freitas et al., 2001). At the end of second week, the highest body mass was achieved in the control group while in the experimental groups with Garlic at 2% was slightly lower (Hernandez et al., 2004). That was probably due to reduced food consumption, resulting from the intense smell of Garlic, which required a period of adaptation of chickens to this kind of feed. Body growth was raised and also developed the meat quality and feed conversion while pigs were fed with 1% Garlic (Cullen et al., 2005).

However, Garlic manifested its stimulating effects and the experimental groups had higher body mass than the control group of chicks at the end of fourth week (Horton et al., 1991). After termination of experiment, Garlic registered a statistically significant increase in body weight in comparison to the control group. In regard to the weight of chicks, separated by gender, the same tendency is observed in both male and female birds at the end of that experiment.

Garlic powder at different levels (0.5 to 3%) had no significant effect on weight gain during the first 21 days of feeding trial compared to that of control birds (Raeesi et al., 2010). However, for the period from 22-42 days, Garlic level at 1% resulted in the highest weight gain. For the whole feeding period Garlic levels of 1% and 3% significantly increased body weight gain as compared with 0.5% Garlic supplemented groups but it was not significant in comparison with control group.

Supplementation of 1% Garlic powder decreased FCR compared with 0.5% supplemented and control group (Raeesi et al., 2010). Birds received 3% Garlic powder in their diets had better FCR than control group. Significantly more feed consumed by control groups rather than the others, except those which were supplemented with 0.5% Garlic powder. There were no significant differences between control and 0.5% supplemented group, although they had lower FCR. Groups which were supplemented with Garlic powder in just the finisher diet had better FCR than those which were supplemented for the whole of the experiment.

Control groups consumed more feed but they had no significant difference with which supplemented with Garlic in starter diet (Raeesi et al., 2010). Addition of Garlic supplementation in diet did not affect growth, feed intake and FCR in whole of the experiment (Demir et al., 2003). Although performance was not affected but when broiler diets were supplemented with 1.5%, 3% and 4.5% Garlic in powder form, their serum and liver cholesterol decreased significantly. They also indicated that this supplementation did not influence FCR (Konjufca et al., 1997). Garlic extract increased body weight gain and also improved FCR in broilers between 7-27 days (Lewis et al., 2003). Broilers received blend of essential oils of Garlic, had higher weight and feed intake and also lower FCR than control group (Alcicek et al., 2003).

2.6.3 Effects of Garlic on blood lipids

Allicin extracted from Garlic had a lipid-lowering effect on long-term feeding to healthy rats (Augusti, 1974). These studies reported a significant decrease in total serum lipids, phospholipids and cholesterol in the animals fed allicin compared to control animals.

Essential oils of Garlic and Onion (equivalent to 1g/kg/day of raw bulbs) with antilipidemic agent, in rabbits fed a diet containing 0.2g cholesterol/kg/day. At the end of 3 months, the serum cholesterol was statistically reduced in the animals receiving Garlic (Bordia et al., 1975). In two separate studies, (Bordia and Verma, 1978; Bordia and Verma, 1980) Garlic extract equivalent to 2g/kg/day of raw Garlic showed a significant decrease in LDL and which was accompanied by more significant increases in HDL. In a later study, increased HDL levels were

demonstrated in rats fed freeze dried Garlic powder. No changes in HDL were noted when the same diet was supplemented with Garlic powder (Kamanna and Chandrasekhara, 1982).

The comparative effects of Garlic and onion were studied in rabbits fed a diet containing cholesterol (0.5 g/day). A steady increase in serum cholesterol was observed in the control group at 8 weeks and 16 weeks. The group receiving Garlic (25g raw/day) showed significantly slow rise in cholesterol at 8 weeks and 16 weeks compared to the control group. The group supplemented with Onion (25g raw/day) showed increases similar to those of the control, reaching maximum cholesterol at 16 weeks (Jain, 1976). The cholesterol-lowering effect of Garlic was also seen in rabbits that were fed 2g cholesterol/day for 16 weeks (Jain and Konar, 1978).

Cholesterol concentrations were not significantly affected by the supplementation of dietary Garlic powder at different levels (0 and 1g/kg) over a 35 days growth period (Horton et al., 1991). Some studies suggested that commercial Garlic oil, Garlic powder and Garlic extract may not be hypocholesterolemic (Chowdhury and Smith, 2002; Berthold et al., 1998; Isaacsohn et al., 1998; McCrindle et al., 1998). Garlic significantly reduced the serum levels of cholesterol, LDL and TG and significantly increased the level of HDL (Rahimi et al., 2011).

2.7 *Azadirachta indica* A. Juss./ *Melia azadirachta* L.

Commonly known as Neem, Nimba, Nimb. More than 140 compounds isolated from different parts of Neem tree (Subapriya and Nagini, 2005). Quercetin and ß-sitosterol, were the first polyphenolic flavonoids purified from Neem fresh leaves and were known to have antibacterial and antifungal properties (Govindachari et al., 1998). Various parts of the plant and the Neem oil contain triterpenoid bitter principles, saponins, flavoniids, tannins and alkaloids. The bitter principles include nimbidin, nimbin, nimbinine, 6-desacetylnimbinine, nimbidol, nimbolide and bakayanin. The bitter constituent nimbin molecule contains an acetoxy, a lactone, an ester of methoxy, and an aldehyde group. The leaves contain azadirachtin, salanin, meliantriol, margosopicrin, paraisine, azadion nine, nimbinene, nimboilde, quercetin and its glycosides, beta sitosterol, hexacosanol, nonacosane and ascorbic acid in amino acids (Vinoth et al., 2012).

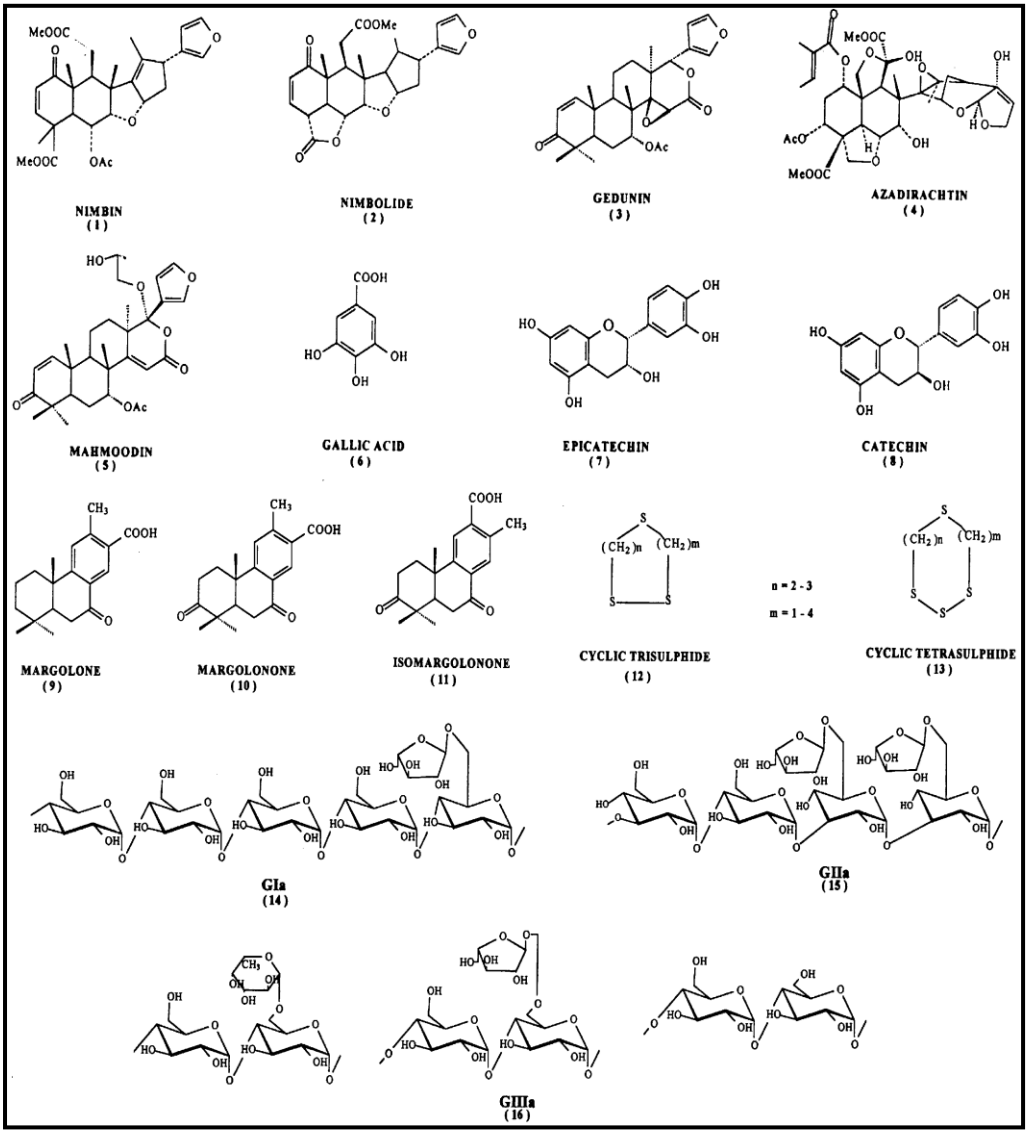


Figure 5: Structure of bioactive Neem compound (Biswas et al., 2002)

2.7.1 Use of Neem plants

Several pharmacological activities and medicinal applications of various parts of Neem are well known. Biological activity of Neem is reported with the crude extracts and their different fractions from leaf, bark, root, seed and oil (Biswas et al., 2002). However, crude extract of different parts of Neem have been used as traditional medicine for the treatment of various diseases. Leaves used as antidermatic, antifungal, anticlotting agent, antihelminthic, antituberculosis, antitumour, antiseptic, fertilizers, insecticides, nematicides and insect repellents (Anonymous, 2006; Sateesh, 1998).

2.7.2 Pharmacological activity

Since time immemorial, people are aware of medicinal properties of Neem. Neem has been extensively used in Ayurveda, Unani and Homeopathic medicine. Neem leaves exhibits a wide range of pharmacological activities (Subapriya and Nagini, 2005).

2.7.3 Antifungal activity

Neem plant is a known inhibitor of fungal growth. The effect of different concentrations of aqueous Neem leaf extract was assayed through TLC on fungal growth and aflatoxin production by *Aspergillus parasiticus* at different incubation times (Ghorbanian et al., 2007). Extracts of Neem leaf, Neem oil and seed kernels are effective against certain fungi, including *Trichophyton*, *Epidermophyton*, *Microsporum*, *Trichosporon*, *Geotricum*, *Aspergillus* and *Candida* (Mondali et al., 2009; Khan et al., 1987). High antimycotic activity with Neem leaf extracts of different parts was recorded (Bansod and Rai, 2008; Jacobson, 1986).

Crude aqueous extracts of Neem (*Azadirachta indica*) were found to have an inhibitory effect on the growth of *A. flavus* and *A. parasiticus* (Thanaboripat et al., 2000). Neem leaf extract prepared by blending fresh leaves (50g) in 1L of 10mM potassium phosphate and added directly to submerged culture of *A. parasiticus* at concentration greater than 10% (v/v) did not affect fungal growth but blocked aflatoxin biosynthesis (Bhatnagar et al., 1990; Bhatnagar, 1988). The tetrano-tri-terpenoids and volatile compounds in Neem are reported to be responsible for its antiaflatoxigenic properties (Choudary, 2002).

2.7.4 Haematological and biochemical effect

The mean values for PCV, Hb and red blood cell in treatments group were not significantly differed from control group (Aruwayo et al., 2011). On the other hand, the white blood cell values in control group were similar to the treatments group. The values for neutrophil, eosinophil and basophil in the control treatment were not significantly different from the Neem treatment except lymphocytes and monocytes. Monocyte count was higher in Neem treated group (Gangar et al., 2006; Borjesson et al., 2000).

In a limited clinical trial, oral administration of 100mg nimbidin three times daily for 10 consecutive days to tropical eosinophilia patients, caused 25% reduction in total eosinophil count with a marked symptomatic relief. For biochemical parameters, level of ALT and creatinine in the control and treated group did not show any significant difference while AST values showed significant difference between the control and Neem treated group (Aruwayo et al., 2011; Musalia et al., 2000). Higher doses of Neem extract showed significant increase in activity of ALT of albino rats (Dafalla et al., 2012). Necrosis of liver, kidney and mild histo-pathological changes on rats were seen in the investigation (Rahman et al., 2001; Bhanwra et al., 2000).

Brown hisex chicks, when fed with a diet containing 2% and 5% Neem leaf from their 7^{th} to 35^{th} day after birth developed hepatonephropathy and significant change in blood parameters. The seed of Neem causes mild to severe changes in kidney, liver, spleen, intestine and heart of chicks (Jacobson, 1995).

2.8 *Ocimum tenuiflorum*/ *Ocimum sanctum* L.

Generally named as Tulsi, Tulasi, Holy Basil, Vishnu Priya. The leaf volatile oil contains eugenol (1-hydroxy-2-methoxy-4-allylbenzene), urosolic acid, carvacrol (5-isopropyl-2-methylphenol), linalool (3, 7-dimethylocta-1, 6-dien-3-ol), limatrol, caryophyllene, methyl carvicol (also called Estragol: 1-allyl-4-methoxybenzene) while the seed volatile oil have fatty acids and sitosterol. Two major sugars of the plants are xylose and polysaccharides (Kumar et al., 2010; Shishodia et al., 2003; Kelm et al., 2000). The aqueous extract of *O. sanctum* leaves revealed alkaloids, flavonoids, tannins and carbohydrates (Aswar and Joshi, 2010; Gupta et al., 2002).

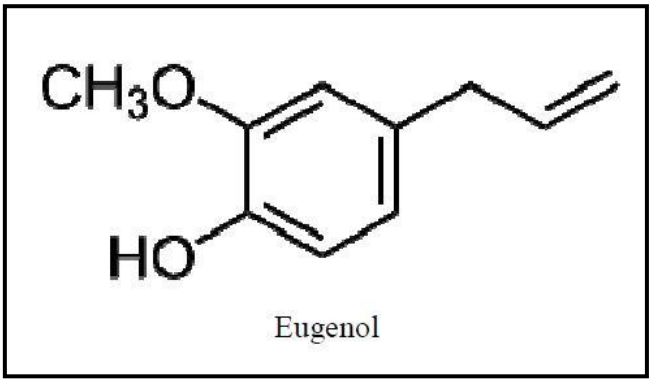


Figure 6: Chemical constituent of tulsi leaf (Singh et al., 2012)

2.8.1 Pharmacological uses

Tulasi is also known as "the elixir of life" since it promotes longevity. Different parts of plant are used in ayurveda and siddha systems of medicine for prevention and cure of many illnesses like common cold, headache, cough, flu, earache, fever, colic pain, sore throat, bronchitis, asthma, hepatic diseases, malaria fever, as an antidote for snake bite and scorpion sting, flatulence, migraine headaches, fatigue, skin diseases, wound, insomnia, arthritis, digestive disorders, night blindness, diarrhea and influenza. The leaves are good for nerves and to sharpen memory. Chewing of leaves also cures ulcers and infections of mouth (Prajapati et al., 2003).

2.8.2 Antifungal activity

Extract of *Ocimum sanctum* effectively worked against some well known fungal agents such as *Candida albicans* (Sharma, 2010; Kaya et al., 2008; Ahmad and Beg, 2001; Geeta et al., 2001), *Fusarium solani* f. sp. Melongenae (Joseph et al., 2008), *Aspergillus flavus* and aflatoxin B_1 (AFB_1) production (Reddy et al., 2009), *Aspergillus niger* (Tewari and Robert, 2009), *Aspergillus repens*, *Curvularia lunata* and *Fusarium moniliforme* (Amadi et al., 2010).

Leaves extracts have been found effective in controlling *Fusarium solani* f. sp. Melongenae (Joseph et al., 2008). Similarly these were also found to inhibit the *A. flavus growth* (65–78%) and AFB_1 production (Reddy et al., 2009). Moreover, when these extracts were interacted with ferrocyanides of Manganese, Silver and Titanium against *Aspergillus niger* the metal ferrocyanides complexes showed more antifungal property in comparison to metal ferrocyanides and extract alone (Tewari and Robert, 2009). Antifungal properties of African Basil (*Ocimum gratissimum* L.) extracts against *Aspergillus repens*, *Curvularia lunata* and *Fusarium moniliforme* using the pour plate method revealed that *A. repens* was more sensitive to *Ocimum* extracts than *C. lunata* and *F. moniliforme* (Amadi et al., 2010).

2.8.3 Haematological and biochemical effect

Ocimum sanctum leaf extract was found to be hepatoprotective against hepatotoxic paracetamol affected rats by significant reduction of AST, ALT, alkaline phosphatase (ALP) and showed marked reduction in fatty degeneration of liver (Chattopadhay et al., 1992). Administration of combination of Tulsi aqueous leaf extract and

gentamicin, significantly prevented rise in serum creatinine and blood urea compared to the only gentamicin treated group in rats (Muglikar et al., 2004). Leaves and seeds of Tulsi plant have been reported to reduce blood and uric acid level in albino rabbits and also possessed diuretic property (Sarkar et al., 1990).

The aqueous extract of Tulsi mixed with diet where significant reduction occurred in blood glucose, serum lipid profile, lipid peroxidation products and improvement in glucose tolerance to diabetic induced rat. The aqueous extract also increased antioxidant enzymes like super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione transferases (GT). It also increased antioxidant like reduced glutathione (GSH) levels in plasma and liver, lung, kidney and brain of rat (Hussain et al., 2001).

Hematological parameters like hemoglobin content, WBC, RBC and differential cell counts were not significantly differed with Tulsi treated rats from control group. Moreover, biochemical parameters like AST, ALT, ALP, creatinine, albumin, blood glucose, total protein, cholesterol and bilirubin did not show any difference with the extract compared to control group (Gautam and Goel, 2014).

2.9 Conclusion

According to literature cited in this chapter it is clearly apparent that plant has been one of the important sources of medicines since the beginning of human civilization. Every parts of plant are used as an antioxidant, antidiabetics, hepatoprotective, antitumor, antifungal, antibacterial, antiviral and anthelmintic agents. A structured and systematic study is authentic in order to assess appropriate antifungal efficacy of medicinal plants (Neem, Tulsi, Onion and Garlic) to control the fungal infection in broilers of Bangladesh. Scientific works on determination of the effect of these plants on heamatological and biochemical parameters as well as growth performance in broilers is also justified to develop a safer and more effective acceptance than synthetically produced antifungal agents.

Chapter-3: Materials and Methods

3.1 Study area and study period

The study was conducted from July to December, 2014 at Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. The experimental farm shed was located at the 2nd floor, opposite to physiology laboratory in academic building no-1, CVASU (Figure 7). The shed was selected based on location, easy convenience to regular data recording and availability of specialized veterinarians.

Figure 7: Study area (CVASU)

3.2 Study design

An experimental study was undertaken to assess comparative efficacy of synthetic antifungals and medicinal plant extract against aspergillosis in broilers (Figure 8).

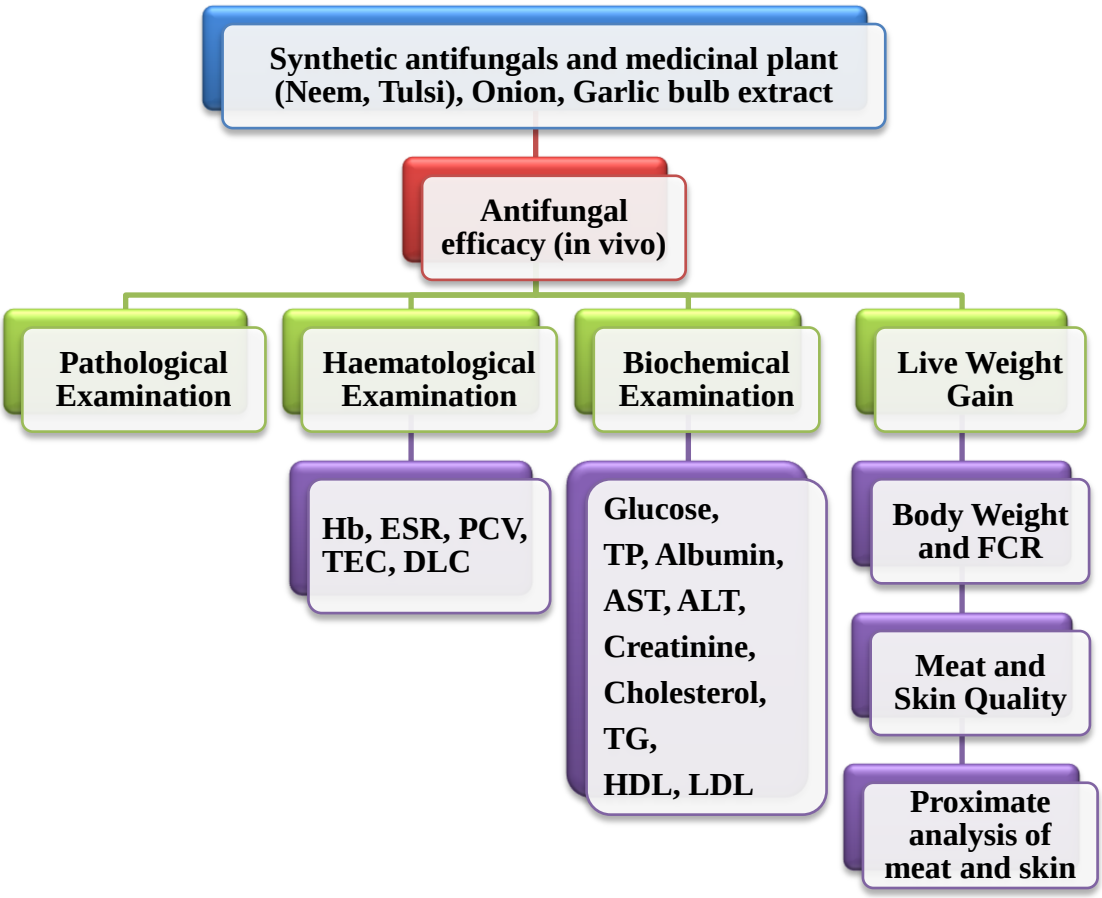


Figure 8: Flow chart of study design

3.3 Medicinal plants used in this study

Scientific name	Local name	Family	Plant part used	Medicinal use
Allium cepa	Onion oil	Amaryllidaceae	Bulb	Enhance immunity, **Antifungal**
Allium sativum	Garlic oil	Liliaceae	Bulb	Expectorant, Antibacterial, **Antifungal**
Azadirachta indica	Neem extract	Meliaceae	Leaves and seeds	Antibacterial, Antiviral, **Antifungal**
Ocimum sanctum	Tulsi extract	Lamiaceae	Leaves	Antibacterial, **Antifungal**

(Bansod and Rai, 2008)

3.4 Collection of plant materials

Fresh leaves of Neem *(Azadirchta indica* A. Juss) and Tulsi (*Ocimum tenuiflorum* or *Ocimum sanctum*) were collected from the medicinal plants garden of CVASU. Fresh Onion (*Allium cepa*) and Garlic (*Allium sativum*) were collected from Bangladesh Agriculture Development Corporation (BADC), Chittagong.

3.5 Drying, grinding and collection of bulbs

Collected plant leaves were thoroughly cleaned by washing and discarding of all the unwanted materials and aged leaves. Then the leaves were air-dried for one week and further dried in oven (Miyako, Japan) at 100°C for half an hour to remove moisture. Dust was made by pulverizing the dried leaves with the help of mortar and pestle. A 25-mesh diameter sieve was used to obtain fine dust and preserved them into airtight plastic bag. On the other hand, collected Onion and Garlic were peeled off all the membrane to elicit the bulb. After bringing out the bulbs, these were kept on airtight plastic container until being used.

3.6 Preparation of Neem and Tulsi extract

Sixty (60) g of Neem leaves dust were taken into two 500ml beaker separately where one beaker consists of 35g Neem leaves dust with 385ml of ethanol and another was 25g Neem leaves dust with 275ml ethanol (1g: 11ml). Then the mixture was stirred for 30 minutes by a magnetic stirrer (1000rpm) and left for 45days. On the other hand, 16.5g of Tulsi leaves dust were taken into 500ml beaker on which all the dust were mixed with 363ml of ethanol (1g: 22ml). Then the mixture was stirred for 30 minutes by a magnetic stirrer (1000rpm) and left for 30 days. All the mixture was filtered through Whatman no.1 filter paper separately. Then the filtrates were taken into a round bottom flask of rotator vacuum evaporator and condensed by evaporation of solvent from filtrate in a water bath at 50°C for ethanol to make a final volume of 5ml. After the evaporation of solvent from filtrate, the condensed greenish extracts were preserved in tightly corked-labeled bottle and stored in a refrigerator (4°C) until further screening for antifungal property (Appendix-A).

3.7 Preparation of Onion and Garlic extract

About 250g Onion bulb was grinded through mixer grinder (Jaipan, India) and squeezed aseptically for collection of onion juice. Following centrifugation at

3000rpm (Hettich Zentrifugen, model: EBA 21, UK), the supernatant was collected and taken into eppendorf tube, stored in a refrigerator (0°C). On the other hand, 500g Garlic bulb was mixed with 50ml distilled waterand the mixture was grinded from beginning to end by using grinder (Jaipan, India). Then the juice was in a tightly corked labeled bottle and stored in a refrigerator (0°C).

3.8 Preparation of *Aspergillus sp.*

Aspergillus spp. isolate was collected from a repository of the Department of Microbiology and Public Health, CVASU. This fungus was inoculated into 5 broiler chicks (3 days old) through drinking water. Two (2) chicks were died and three (3) chicks showed symptoms of gasping, rapidly breathing, snoozing and drowsiness at the 2nd day of post inoculation. Post mortem examination of dead chicks revealed characteristic "sagu" nodules at lungs (Figure 9), air sac and pericardium. These collected samples were inoculated onto Saboured Dextrose Agar (SDA) (Oxoid) and growth of fungi, hyphal tips and spores were observed (Figure 9) after incubation for 3 days at 30°C (Moss, 1998). Pure cultures of *Aspergillus spp.* were obtained by transferring selected colonies from isolated medium and repeated sub-culturing (Moss, 1998). This isolated strain of *Aspergillus spp.* was used for experimental purposes in the study.

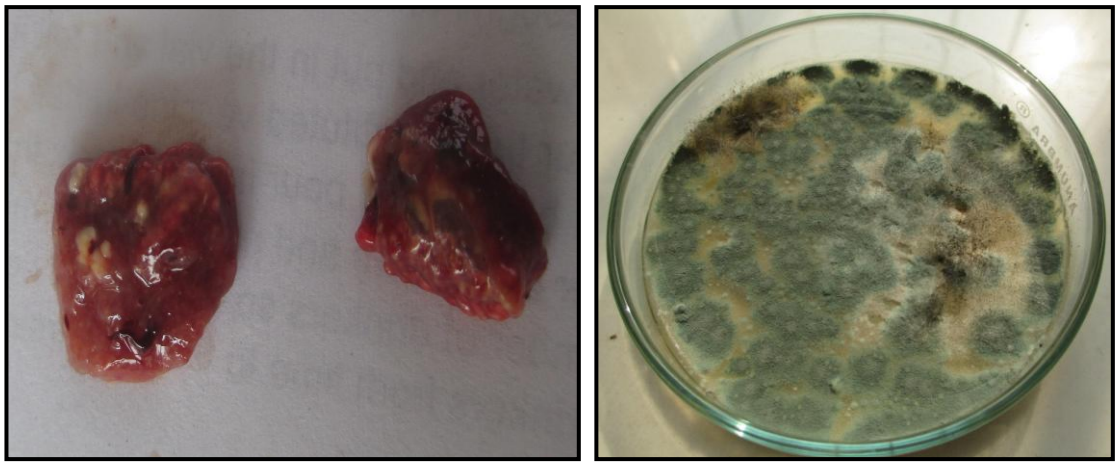


Figure 9: Nodules in lungs (Left figure) and growth of *Aspergillus sp.* on SDA (Right figure)

3.9 Experiment

In vivo antifungal trial was performed on ethanol extracted neem and tulsi leaves and onion and garlic bulb comparing with synthetic antifungals such as 0.1% $CuSO_4$ and

Nystatin Sulphate in broilers. The effect on meat and skin as well as pathological findings and certain haemato-biochemical parameters were also observed.

3.9.1 Selection of chicks as study population

A total of 150 day-old "A" grade healthy chicks (Cobb-500) were purchased from M. M. Agha Poultry (Raufabad, Oxygen, Chittagong) as pre-contract basis for this study.

3.9.2 Management of chicks

Chicks were placed in a chick guard (brooding house) and during brooding period, temperature was maintained as 95°F and humidity was 65% for the 1st week and for the next week, temperature was 88-90°F and humidity was 70%. Commercially available starter feeds (M. M. Agha Poultry) were provided for 13 days (2950 Kcal/Kg ME; 22.7% CP). From 14 to 26 days, they were given manually mixed balanced mash feed (3080Kcal/Kg ME and 21% CP) (Appendix-B) and after 26 days, again provided the readymade pellet feed (Anchor Special, Elia feeds Ltd, Sector-9, Uttara, Dhaka) which containing 3050 Kcal/Kg ME and 22% CP. Grower chicks were housed into cages according to their groups of treatment intervention at Day-11. Appropriate vaccination schedule was maintained against different viral diseases such as Marek's vaccine at Day-0, Baby Chick Ranikhet Disease Vaccine (BCRDV) at Day-3 and Day-22 and Gumboro vaccine at Day-11 (Figure 10B). Everyday *ad libitum* water mixed with various vitamins, minerals, electrolytes, enzymes, phytozyme, probiotics, extract of *Yucca schidigera*, glucose and saline were provided.

3.9.3 General monitoring task

On the brooding period, chicks were monitored every two hours interval, checked the temperature and humidity of chick guard. The floor of chick guard was maintained by rice husk and paper materials (Figure 10A, 10C). The papers were replaced with new paper every twelve hours interval for first 5 days of brooding and only rice husk was used as bedding material and changed every 24 hours interval until Day-11. Droppings of the birds from cages were carefully removed by washing the tray once in a day. Proper ventilation was maintained for ensuring the comfort of the birds.

Figure 10: A. Brooding of chicks, B. Vaccination of birds (BCRDV vaccine, Day-3), C. Rice husk as bedding material, D. Birds in cages

3.9.4 Treatment intervention

The seven bird groups (T_0-T_6) were designed as follows (Figure 11). Each bird group consists of 21 birds except T_3 (20 birds).

Figure 11: Layout of treatment intervention

3.9.5 Infection exposure

The infection of *Aspergillus spp.* was introduced at Day-16 to all birds (T_1 to T_6 group) through mash feed and water. Infection dose was 0.2g/kg on feed and 1.25mg/ml on water according to Moss, 1998 (Figure 12). At Day-20, 3 sub-groups were created within each group and re-infection was introduced through different concentration of water (high 2.75mg/ml, medium 2.13mg/ml and low 1.5mg/ml).

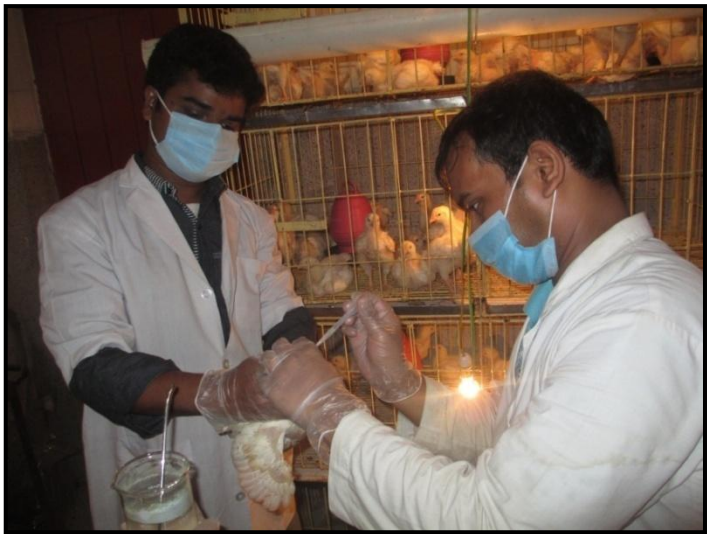


Figure 12: Challenged the birds through *Aspergillus spp.*

3.9.6 Diagnosis by clinical signs after infection

Aspergillosis infected broilers were suffered in gasping, suffocation, dyspnoea, coughing and mucus through nostrils and infected birds were sitting silently other than normal birds, did not want to ingest feed and had a huge thirst (Figure 13). Post mortem findings of dead birds revealed the characteristics whitish/yellowish "sagu" nodules at lungs, air sac, pericardium, heart and other different organs; congested lung.

Figure 13: Infected birds with suffocation, dyspnoea with dizziness

3.9.7 Confirmation by histopathology

Periodic Acid-Schiff (PAS) stain technique (Appendix-C) was performed to identify the histopathological findings. Lungs revealed classical granulomatous lesions containing huge macrophages, mononuclear inflammatory cells and radically arranged foreign body type giant cells (Figure 14). Fungal elements are observed in eosinophilic cytoplasm of multinucleated cells and lymphocytes infiltrate margin of the granuloma. Progressive inflammation in lungs resulting in small granuloma coalescence causes more extensive lesions with necrotic eosinophilic material containing congested erythrocytes, degenerated heterophils and exfoliated epithelial cells (Figure 15).

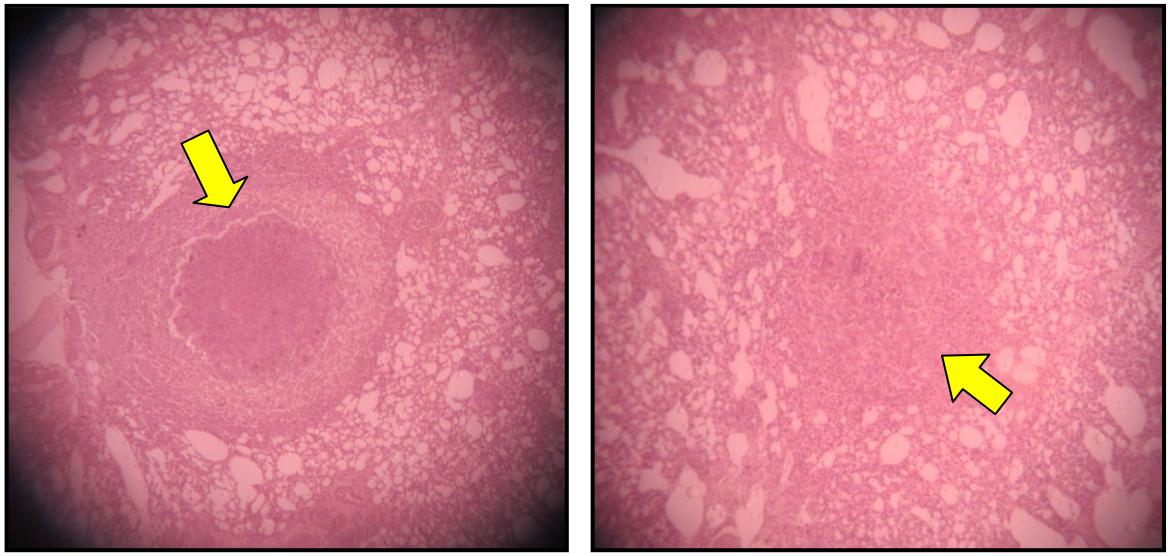


Figure 14: Granulomatous lesions radically arranged foreign body type giant cells (arrow marks indicate granuloma)

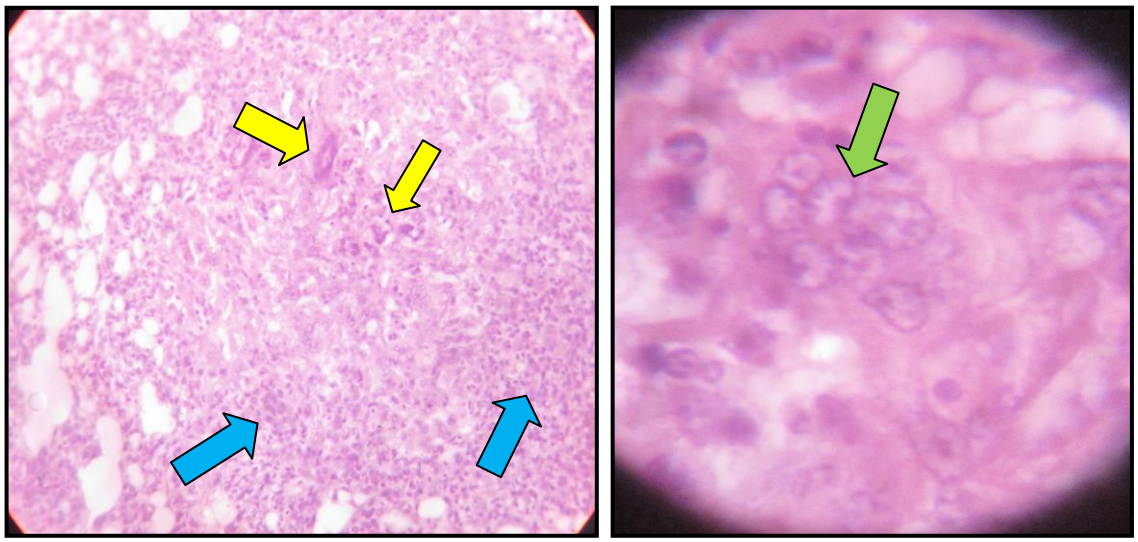


Figure 15: Huge macrophages (Yellow arrows), infiltrative mononuclear cells (Blue arrows) and foreign body type giant cells (Green arrow)

3.9.8 Treatment and post monitoring

After showing sign-symptom, treatment was started from 22nd day age of the birds. In groups (T_1 to T_6), birds were treated according to their treatment lay out (Figure 11). On every group except T_0 group 3ml drugs were administered through water where dosing as Nystatin Sulphate (100000IU/ml), 0.1% $CuSO_4$ (1mg/ml), Neem (90.91mg/ml), Tulsi (45.46mg/ml), Garlic (1667mg/ml) and Onion (7576mg/ml) and continued up to Day-27 (Figure 16).

Figure 16: Synthetic antifungals and plant extracts used in this study

3.9.9 Collection and preservation of sample

Blood sample was collected from jugular vein (Figure 17) and 1.5ml blood was placed into vacutainer containing anticoagulant and 1.5ml blood was placed in another vacutainer without anticoagulant. Every sample on each vacutainer was given unique identity number and group name. Blood was collected at Day-18, Day-25 and Day-30 to determine hematological parameters such as ESR, PCV, Hb, TEC, DLC and biochemical parameters such as Glucose, TP, Albumin, HDL, LDL, TG, Cholesterol, ALT, AST and Creatinine. After collection, samples were immediately

shipped to physiology laboratory of CVASU for hematological evaluation and research laboratory of CVASU for serum separation and biochemical analysis.

Figure 17: Blood collection (Left figure) and sacrificing one bird from each group at Day-30 (Right figure)

3.9.10 Drug efficacy through FCR calculation

Initially, every chick was weighed to measure appropriate growth and then randomly 15 chicks (10%) were weighed at 7 days interval. Feed Efficiency or Feed Conversion Ratio (FCR) was calculated according to the following formula-

$$\text{Feed efficiency or FCR} = \frac{\text{Unit of feed consumed}}{\text{Unit of weight increased}}$$

3.9.11 Evaluation of hematological parameters

EDTA containing blood samples were used to determine the hematological parameters such as ESR, PCV, Hb, TEC and DLC at day 18, day 25 and day 30.

3.9.11.1 Erythrocyte Sedimentation Rate (ESR)

Collected blood samples were filled with the special loading pipette. The tip of the pipette was inserted to the bottom of the hematocrit tube/wintrobe tube and blood was expelled by the pressure on the rubber bulb. ESR value was determined according to the method explained by Coffin (1955).

3.9.11.2 Haematocrit or Packed Cell Volume (PCV)

The haematocrit tube was filled up with well-mixed blood by special loading pipette. Then the tube was centrifuged at 3000rpm for half an hour and reading was taken. The PCV was determined as per method described by Coffin (1955).

3.9.11.3 Hemoglobin (Hb) concentration

The Hb estimation was performed by the acid Hematin method with the Shali's Hemometer. The result was read as per method described by Coffin (1955).

3.9.11.4 Total Erythrocytes Count (TEC)

Thomas red blood cell pipette was used and pipette was filled up to 0.5 marks with blood and diluting fluid (Hayem's solution). The dilution of the contents was 1:200. The counting chamber was filled with the contents and was placed under microscope. Counting of cells and calculation of TEC was performed as per method indicated by Coffin (1955).

3.9.11.5 Differential Leucocytes Count (DLC)

A thin blood film was made by spreading a blood drop evenly on clean grease free slide and modification of Romanowsky's stain namely Leishman's stain was used. For Giemsa's staining the air dried blood smears were prefixed with acetone free methanol for five minutes (Conn and Darrow, 1960). The DLC results obtained were compared with the estimated normal values of Simmons et al. (1974) and Schalm et al. (1975).

3.9.12 Separation of serum

After 24 hours the coagulated blood which was kept in vacutainer where no anticoagulant was centrifuged (Capp Rondo, CR-68X, Denmark) for 20 minutes at 3000rpm. Obtained serum samples were shifted to the eppendorf tube by using micropipette and given unique identification no. The obtained serum samples were stored in -20^{o}C until analysis for biochemical test.

3.9.13 Biochemical assay of serum sample

Serum and all reagents were thawed by keeping at room temperature approximately for 30 minutes before the analysis. The serum samples were vortexed for mixing

component of serum uniformly. The Serum Glucose, Total Protein (TP), Albumin (Alb), Triglyceride (TG), Cholesterol, HDL, LDL, AST, ALT, Creatinine (Appendix-D) were assayed by using automated Biochemical analyzer (Humalyzer-3000, Germany) following manufacturers instruction. Randox kits were used to determine glucose, TP, Alb, TG, Cholesterol, AST, ALT, Creatinine; Biorex kits for HDL, LDL. A total number of 63 serum samples were analyzed.

3.9.13.1 Carbohydrate assay (Serum Glucose)

Colorimetric spectrophotometric methods were used for determination of glucose Concentrations (Barham and Trinder, 1972).

3.9.13.2 Total Protein assay

Colorimetric spectrophotometric methods were used for determination of albumin and total protein (Doumas et al., 1975).

3.9.13.3 Albumin assay

Albumin is based on the principle of competitive bindings between albumin and albumin reagent. Bromocresol green forms with albumin in citrate buffer a colored complex. The absorbance of this complex is proportional to the albumin concentration in the sample.

3.9.13.4 Lipid profile

3.9.13.4(a) Cholesterol assay

Colorimetric spectrophotometric methods were used for determination of cholesterol concentrations (Roeschlau et al., 1974).

3.9.13.4(b) Triglyceride assay

Triglycerides were determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4- aminophenezone and 4-Chlorophenol under the catalytic influences of peroxidease.

3.9.13.4(c) Low Density Lipoprotein (LDL) assay

LDL is based on the principle of competitive bindings between LDL and LDL reagent. Low density lipoproteins are precipitated by the addition of heparin at their

isoelectric point (PH 5.04). The HDL and LDL remain in the supernatant and can be determined by enzymatic methods.

LDL Cholesterol = Total Cholesterol – Cholesterol in the supernatant. The absorbance of this complex is proportional to the LDL concentration in the sample.

3.9.13.4(d) High Density Lipoprotein (HDL) assay

Low density lipoprotein (LDL and VLDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotangstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL (high density Lipoprotein) fraction, which remains in the supernatant, was determined.

3.9.13.5 Liver function tests [Aspartate aminotransferase (AST) and alanine aminotransferase (ALT)]

AST and ALT activity was determined according to the method described by Reitman and Frankel (1957).

3.9.13.6 Creatinine concentration

Creatinine was determined by the method described by Husdan and Rapoport (1968).

3.10 Statistical analysis

Data were entered into a spread sheet of MS Excel-2007. The data were sorted, cleaned and coded using the excel program before exporting to STATA/IC-11.0 (STATA Corp, USA) for analysis. A one way ANOVA (Analysis of Variance) was performed to compare the result of different groups according to weight, hematological and biochemical parameters. p values of ≤ 0.05 were considered statistically significant.

Chapter-4: Results

This experiment was conducted to evaluate the antifungal efficacy of 0.1% $CuSO_4$, Nystatin Sulphate, Onion, Garlic, Neem and Tulsi on the basis of growth performance, biochemical and hematological alterations.

4.1 Effects of synthetic antifungals and plant extracts on growth performances

4.1.1 Live weight

During the experiment, increased live weight was observed in all groups of broilers. The highest body weight was observed on Garlic group (1354g ± 76) and lowest body weight was recorded on Tulsi group (1166g ± 82) at termination of the study (Figure 18). In the present study, a significant ($p \leq 0.05$) variation was present on body weight at day 18-24 in treated groups comparing to the control group. Highly significant ($p \leq 0.01$) variation was evident in every group on different observational periods (initial, 0-10 days, 11-17 days, 18-24 days and 25-30 days) of the study (Table 2).

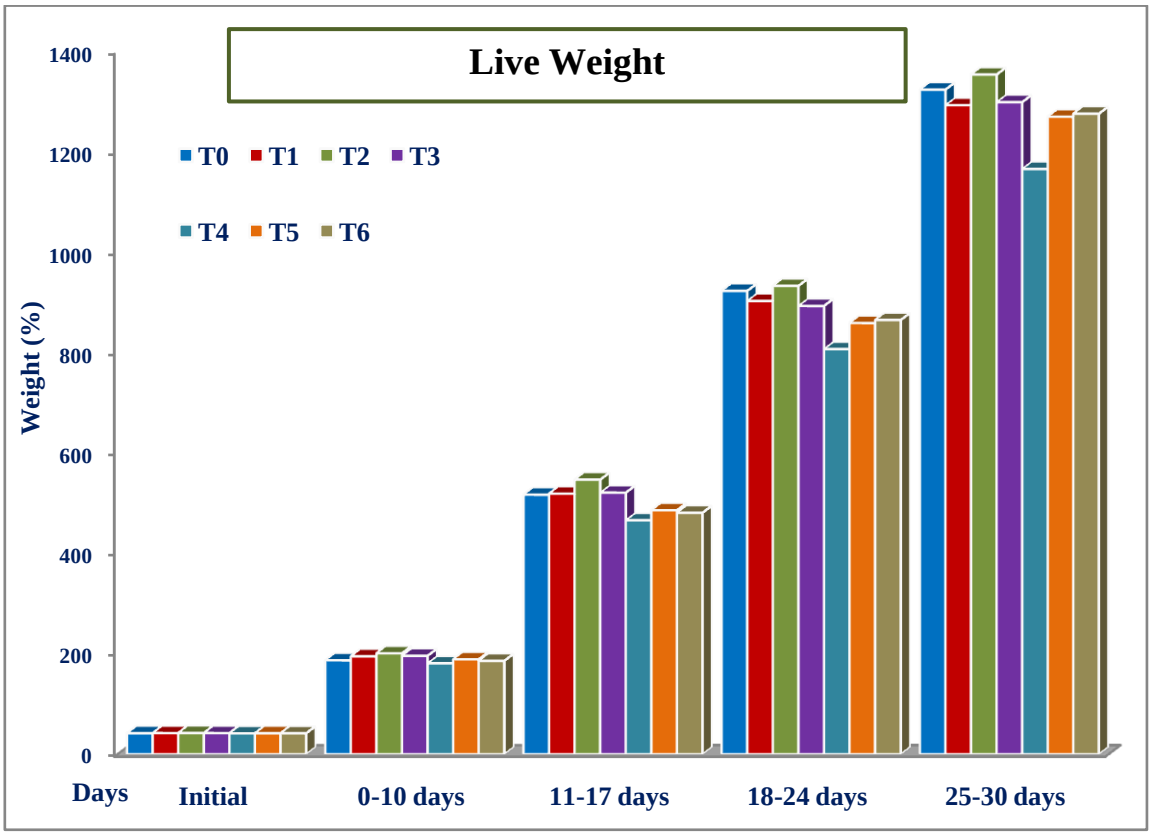


Figure 18: Live weight variation among control and different treated groups

4.1.2 Total feed intake

Total feed intake of different treated or control groups of broiler were illustrated on Table 2. Average feed intake was 210.7g by every chick during the brooding period (0-10 days). Feed intake was increased and did not differ significantly (p >0.05) among the experimental groups. However, the lowest feed intake (2136.3g) was observed in T_1 group and the highest feed (2288.8g) intake was recorded in T_3 group upto termination of the study (Figure 19). In every group, intake of feed differ significantly ($p \leq 0.01$) throughout the observational period of the study.

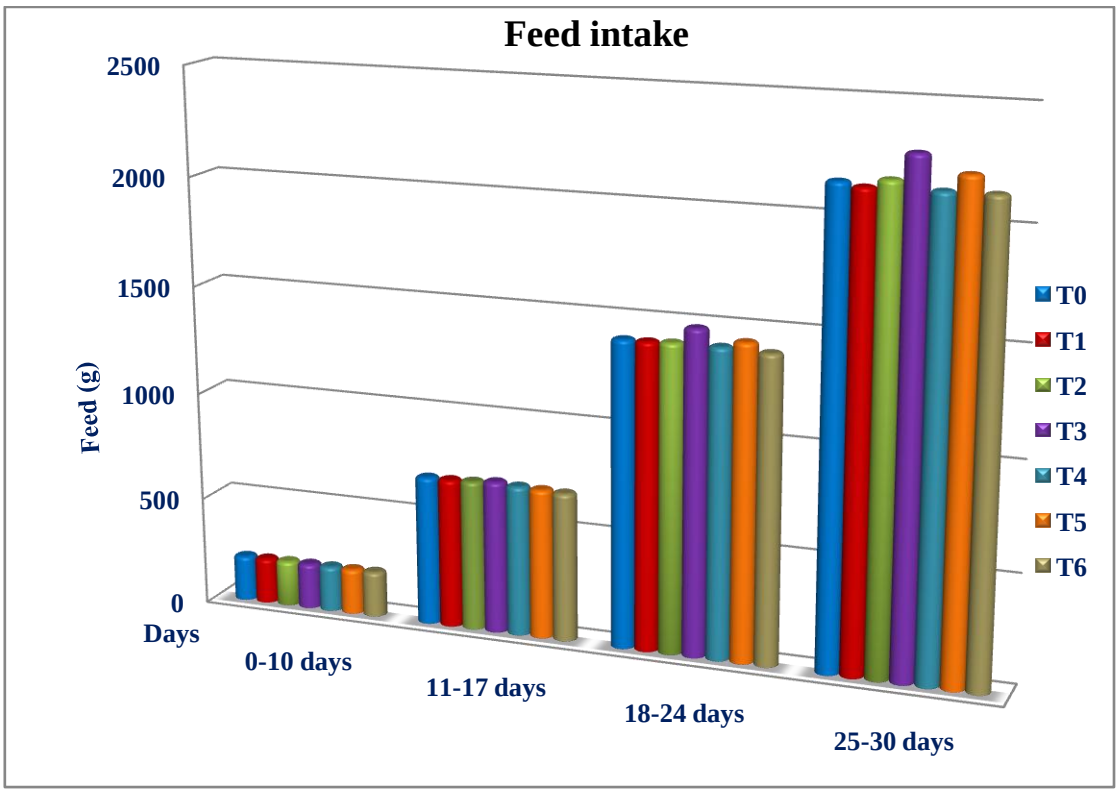


Figure 19: Feed intake variation among control and different treated groups

4.1.3 FCR on live weight

FCR of different stages under various experimental groups are shown in Table 2. It was observed that, FCR were ranging from 1.33 to 1.46 (except Tulsi FCR 1.51) upto age of 10 days and had high significant ($p \leq 0.01$) variation. Among the groups, significant deviation ($p \leq 0.05$) was also evident on 11-17 days, 18-24 days and 25-30 days. Highest FCR was recorded in Tulsi (1.90) and lowest in Garlic (1.66) at the final stage of the study (Figure 20). Moreover, in every group, FCR varied significantly ($p \leq 0.01$) in the study.

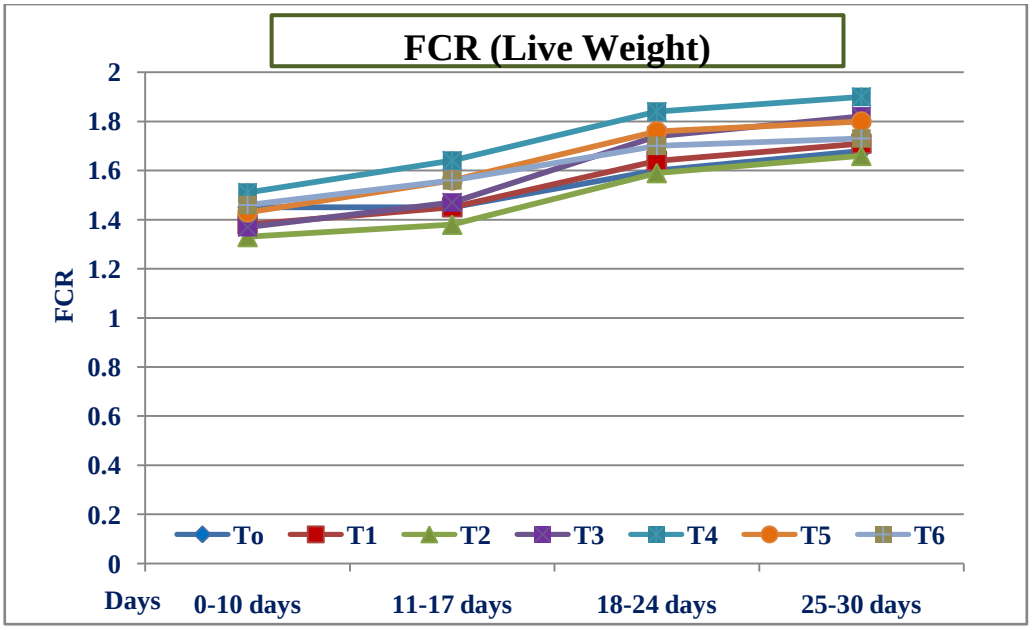


Figure 20: FCR on live weight among control and different treated groups

4.1.4 Weight gain

Initial, body weight of birds were ranged from 40-45g and every seven days interval weight gain was recorded (Table 2) where the findings were insignificantly (p >0.05) differed among the treated groups (Figure 21).

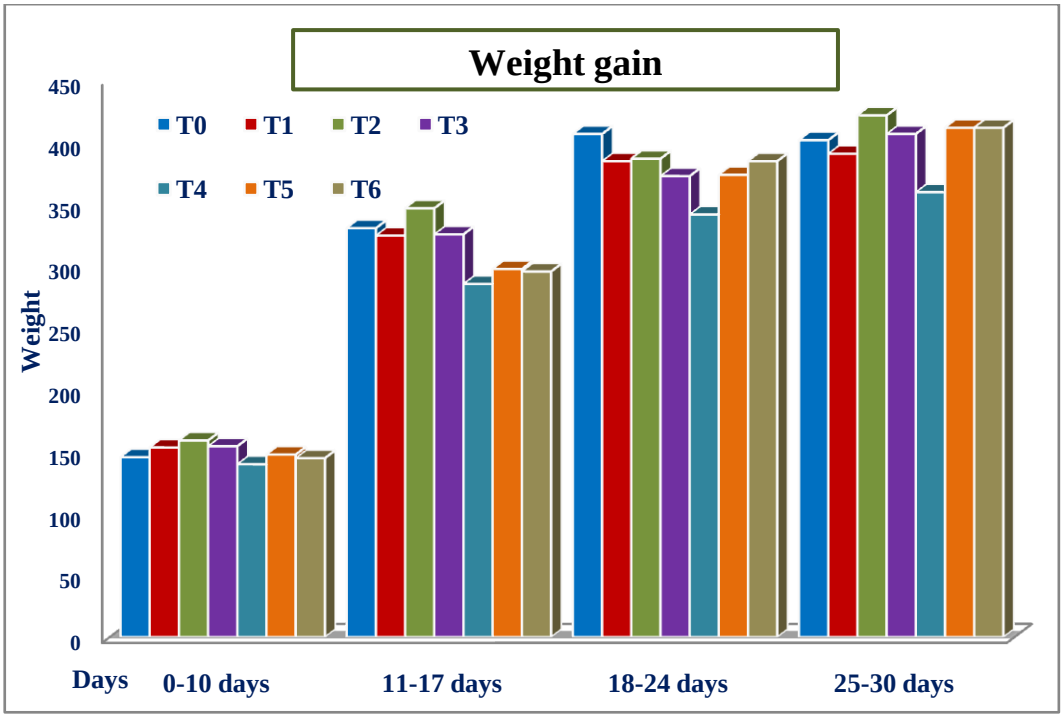


Figure 21: Comparison of weight gain among control and different treated groups

Table 2: Effects of synthetic antifungals and plant extracts on growth performances in broilers affected with aspergillosis

Para-meters	Periods	Groups							*p*
		T_0	T_1	T_2	T_3	T_4	T_5	T_6	
Live Weight (g)	P-1 (Mean ± SD)	41.8 ± 0.8	42 ± 1	42.3 ± 2.4	42 ± 3.2	41.5 ± 1.1	41.7 ± 2.3	41.6 ± 1.9	0.99
	P-2 (Mean ± SD)	187 ± 19.9	195 ± 17.7	201 ± 21.6	196 ± 11.4	181 ± 17.8	189 ± 15.2	186 ± 11.9	0.55
	P-3 (Mean ± SD)	517 ± 27.3	519 ± 29.2	547 ± 54.9	521 ± 49.8	466 ± 52.8	486 ± 27	481 ± 31.3	**0.05**
	P-4 (Mean ± SD)	923 ± 84.1	903 ± 109.7	933 ± 98.5	893 ± 57.8	807 ± 86.1	859 ± 71.4	865 ± 69.3	0.28
	P-5 (Mean ± SD)	1324 ± 63.5	1293 ± 156.6	1354 ± 76	1299 ± 136	1166 ± 82	1270 ± 81.9	1276 ± 67.3	0.15
	p	**< 0.001**	**0.00**	**< 0.001**	**0.002**	**< 0.001**	**0.001**	**0.002**	
Total Feed Intake (g)	P-1 (Mean ± SD)	-	-	-	-	-	-	-	-
	P-2 (Mean ± SD)	210.7 ± 4.5	210.7 ± 4.5	210.7 ± 8.6	210.7 ± 6.2	210.7 ± 9	210.7 ± 10.7	210.7 ± 9.2	1.00
	P-3 (Mean ± SD)	691.2 ± 49.5	691.2 ± 36.6	695.2 ± 52.8	704.3 ± 63.9	695.3 ± 43	691.2 ± 36.4	686.6 ± 23.8	0.99
	P-4 (Mean ± SD)	1408 ± 88.9	1408 ± 22.4	1412 ± 33.6	1484 ± 78.5	1412 ± 58	1441.7 ± 171	1403.4 ± 75	0.91
	P-5 (Mean ± SD)	2154 ± 65.2	2136.3 ± 44.4	2173 ± 100.4	2288.8 ± 67	2140 ± 68.6	2215 ± 42.4	2141.2 ± 75	0.13
	p	**0.000**	**0.01**	**0.003**	**0.000**	**0.02**	**0.02**	**0.009**	
FCR (Live Wt)	P-1 (Mean ± SD)	-	-	-	-	-	-	-	-
	P-2 (Mean ± SD)	1.45 ± 0.01	1.38 ± 0.03	1.33 ± 0.01	1.37 ± 0.01	1.51 ± 0.01	1.43 ± 0.02	1.46 ± 0.02	**0.001**
	P-3 (Mean ± SD)	1.45 ± 0.03	1.45 ± 0.03	1.38 ± 0.03	1.47 ± 0.03	1.64 ± 0.03	1.56 ± 0.02	1.56 ± 0.01	**0.01**
	P-4 (Mean ± SD)	1.60 ± 0.02	1.64 ± 0.02	1.59 ± 0.03	1.74 ± 0.03	1.84 ± 0.03	1.76 ± 0.02	1.70 ± 0.03	**0.001**
	P-5 (Mean ± SD)	1.68 ± 0.03	1.71 ± 0.03	1.66 ± 0.03	1.82 ± 0.03	1.90 ± 0.03	1.80 ± 0.01	1.73 ± 0.03	**0.04**
	p	**< 0.001**	**0.009**	**0.001**	**0.000**	**0.002**	**0.003**	**0.002**	
Weight gain (g)	P-1 (Mean ± SD)	-	-	-	-	-	-	-	-
	P-2 (Mean ± SD)	145.2 ± 20.5	153 ± 16.9	158.7 ± 22	154 ± 11.2	139.5 ± 17.2	147.3 ± 16.4	144.4 ± 12.3	0.60
	P-3 (Mean ± SD)	330 ± 26.2	324 ± 32.9	346 ± 54.2	325 ± 55.7	285 ± 58.6	297 ± 35.3	295 ± 22.6	0.27
	P-4 (Mean ± SD)	406 ± 95.1	384 ± 120.5	386 ± 137.8	372 ± 68.1	341 ± 94.5	373 ± 85.4	384 ± 74.3	0.97
	P-5 (Mean ± SD)	401 ± 130.5	390 ± 254.9	421 ± 143.8	406 ± 185.6	359 ± 145.9	411 ± 121.4	411 ± 78.6	0.99
	p	**0.004**	**0.001**	**0.005**	**0.007**	**0.007**	**0.003**	**0.000**	

Feed consum-ption (g)	P-1 (Mean ± SD)	-	-	-	-	-	-	-	-
	P-2 (Mean ± SD)	210.7 ± 4.5	210.7 ± 1.8	210.7 ± 8.6	210.7 ± 6.2	210.7 ± 9	210.7 ± 10.7	210.7 ± 9.2	1.00
	P-3 (Mean ± SD)	480.5 ± 26.4	480.5 ± 38.2	484.5 ± 31.9	493.6 ± 26.6	484.5 ± 22.9	480.5 ± 23.8	475.9 ± 17.9	0.99
	P-4 (Mean ± SD)	716.8 ± 23.6	716.8 ± 18.7	716.8 ± 14.1	779.6 ± 22.2	716.8 ± 36	750.4 ± 38.3	716.8 ± 35	0.09
	P-5 (Mean ± SD)	746 ± 48.4	728.2 ± 25.5	760.6 ± 35.5	804.9 ± 32.5	728.2 ± 16.4	773.4 ± 34.3	737.8 ± 43.8	0.15
	p	**0.01**	**0.04**	**< 0.001**	**0.004**	**0.000**	**0.001**	**0.03**	
FCR (Wt gain)	P-1 (Mean ± SD)	-	-	-	-	-	-	-	-
	P-2 (Mean ± SD)	1.45 ± 0.01	1.38 ± 0.03	1.33 ± 0.01	1.37 ± 0.01	1.51 ± 0.01	1.43 ± 0.02	1.46 ± 0.02	**0.001**
	P-3 (Mean ± SD)	1.46 ± 0.02	1.48 ± 0.03	1.40 ± 0.02	1.52 ± 0.03	1.70 ± 0.02	1.62 ± 0.01	1.66 ± 0.02	**0.002**
	P-4 (Mean ± SD)	1.77 ± 0.03	1.87 ± 0.02	1.86 ± 0.04	2.10 ± 0.04	2.10 ± 0.05	2.01 ± 0.03	1.87 ± 0.03	**0.000**
	P-5 (Mean ± SD)	1.86 ± 0.04	1.87 ± 0.04	1.81 ± 0.03	1.98 ± 0.03	1.98 ± 0.03	1.88 ± 0.04	1.80 ± 0.03	**0.01**
	p	**0.001**	**0.004**	**0.002**	**<0.001**	**0.001**	**0.003**	**0.000**	

Initial day (P-1), 0-10 days (P-2), 11-17 days (P-3), 18-24 days (P-4) and 25-30 days (P-5)

T_0= Control group, T_1= Onion group, T_2= Garlic group, T_3= Neem group, T_4= Tulsi group, T_5= 0.1% $CuSO_4$ group and T_6= Nystatin Sulphate group

SD= Standard Deviation

Significant variation ($p \leq 0.05$) and Highly Significant variation ($p \leq 0.01$)

4.1.5 Feed consumption

Every chick was taken same amount feed (average 210.7g) on 0-10 days of age and also during the age between 11-17 days (average 475.9g to 493.6g) (Figure 22). However, between 18-24 days and 25-30 days, feed ingestion was varied increasingly (p >0.05) (Table 2). On the other hand, highly significant (p ≤0.01) deviation was evident in every group of the study.

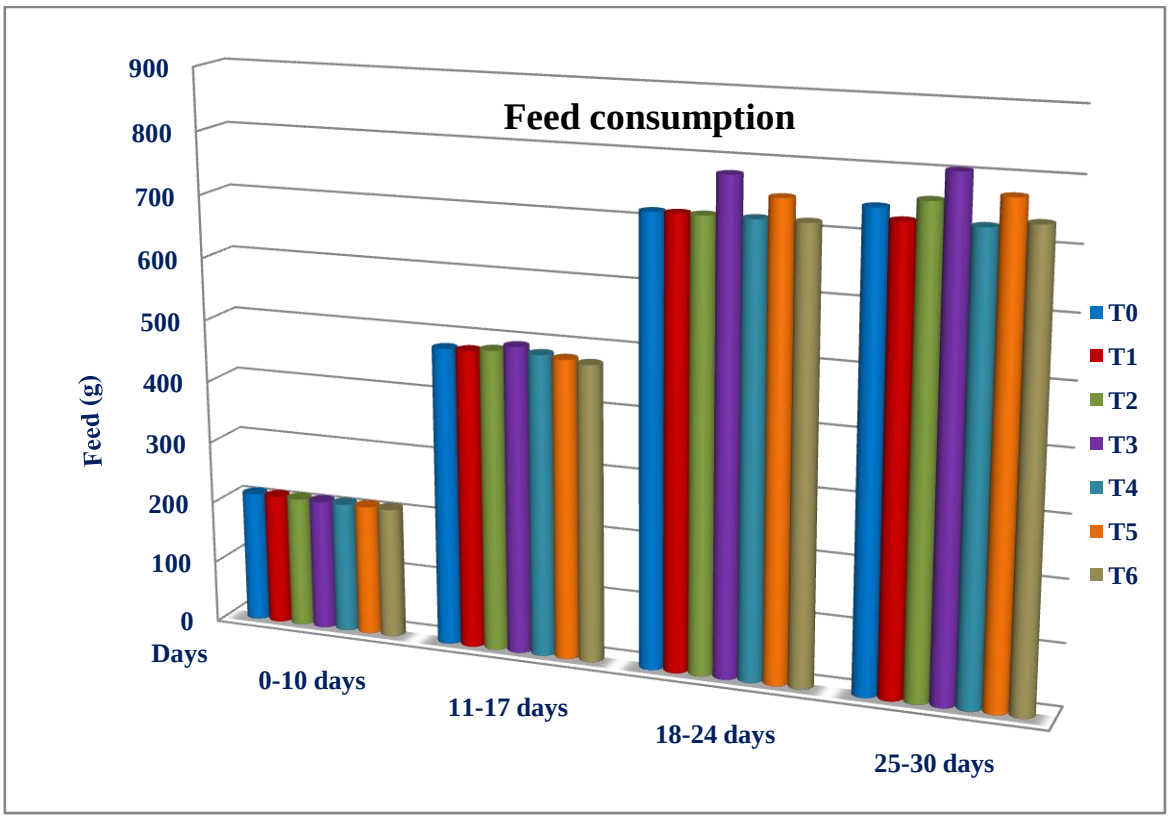


Figure 22: Feed intake variation among control and different treated groups

4.1.6 FCR on weight gain

FCR was rapidly changed on weight gain and significantly (p ≤0.01) altered up to 25-30 days among the groups comparing to control group (Table 2). Moreover, within groups, FCR was reached to the highest at 2.10 in Neem and Tulsi group (Figure 23) where highly significant (p ≤0.01) variation was evident in the study.

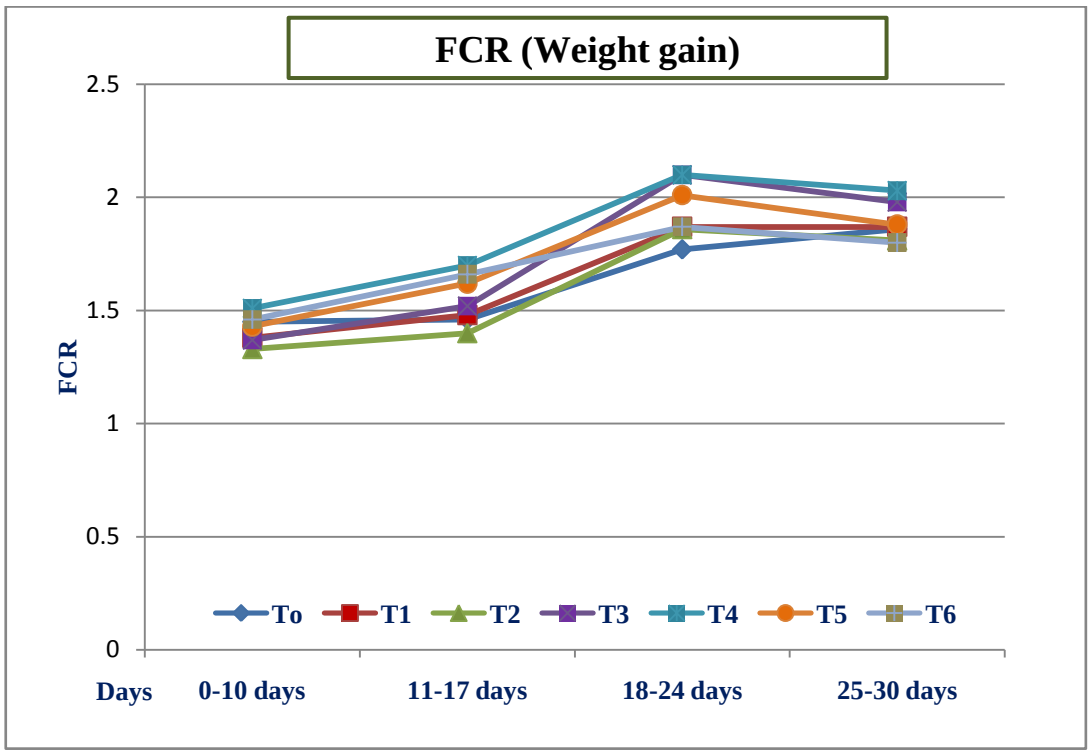


Figure 23: FCR (Weight gain) among control and different treated group

4.2 Effects of synthetic antifungals and plant extracts on blood parameters

4.2.1 Erythrocyte Sedimentation Rate (ESR)

Significant ($p \leq 0.05$) variation was observed in ESR (mm/1st hr) among the treated groups at post treatment period. The mean values of ESR were ranged from 1.7 to 2.5 after infection exposure (Table 3). However, the result was similar during the treatment period which was slightly increased to 3.5 at Tulsi on post treatment stage.

4.2.2 Packed Cell Volume (PCV)

PCV (%) level insignificantly ($p > 0.05$) increased among the treated groups (except T_4 and T_5). On T_4 group, PCV level was decreased from 33.7 to 29 and on T_5 group, it was varied from 32.3 to 31.3 at different observational periods of the study (Table 3).

4.2.3 Hemoglobin (Hb) concentration

Hb (g/dl) content varied significantly ($p \leq 0.05$) at infection exposure and treatment period among the groups of the study (Table 3). In 0.1% $CuSO_4$ group (T_5), Hb concentration was also decreased significantly.

Table 3: Effects of synthetic antifungals and plant extracts on ESR, PCV, Hb and TEC in broilers affected with aspergillosis

Para-meters	Periods	Groups							*p*
		T_0	T_1	T_2	T_3	T_4	T_5	T_6	
ESR (mm/1st hour)	D-1 (Mean ± SD)	1.7 ± 0.3	1.8 ± 1	1.8 ± 0.8	1.7 ± 0.6	2.2 ± 0.8	2.5 ± 1	2 ± 0.5	0.81
	D-2 (Mean ± SD)	2.2 ± 0.8	2.7 ± 0.8	2.5 ± 0.5	1.8 ± 0.8	2.2 ± 0.8	2.8 ± 0.3	2.2 ± 0.8	0.60
	D-3 (Mean ± SD)	1.5 ± 0.5	2 ± 0.5	2.7 ± 0.6	1.8 ± 0.3	3.5 ± 1.3	1.8 ± 0.3	1.7 ± 0.3	**0.02**
	p	0.37	0.46	0.30	0.92	0.24	0.22	0.56	
PCV (%)	D-1 (Mean ± SD)	31.7 ± 2.1	31.7 ± 4.2	30.7 ± 1.5	30.7 ± 3.1	33.7 ± 2.1	32.3 ± 1.5	30.7 ± 2.1	0.74
	D-2 (Mean ± SD)	31.3 ± 3.5	31.3 ± 2.1	33.3 ± 2.5	31.7 ± 2.1	32 ± 3	33.3 ± 0.6	33.7 ± 1.5	0.75
	D-3 (Mean ± SD)	34 ± 1.7	32 ± 1.7	33.3 ± 1.5	32.3 ± 2.1	29 ± 4.4	31.3 ± 2.5	31.7 ± 1.5	0.30
	p	0.43	0.96	0.22	0.72	0.29	0.42	0.18	
Hb (g/dl)	D-1 (Mean ± SD)	9.2 ± 0.3	10.5 ± 1.1	7.3 ± 1.2	9.7 ± 0.7	9.5 ± 1.7	10.8 ± 0.7	8.2 ± 0.4	**0.009**
	D-2 (Mean ± SD)	8.1 ± 0.4	9.6 ± 0.7	8.6 ± 0.7	9.7 ± 1.2	8.9 ± 1.2	11.2 ± 0.5	8.8 ± 1.3	**0.02**
	D-3 (Mean ± SD)	8.5 ± 2.8	9.2 ± 0.2	6.4 ± 2.9	9.1 ± 1.3	8.5 ± 1.7	9 ± 0.6	7.1 ± 0.2	0.38
	p	0.72	0.20	0.40	0.76	0.73	**0.01**	0.11	
TEC (million /mm3)	D-1 (Mean ± SD)	2.4 ± 0.3	2.3 ± 0.1	2.1 ± 0.1	2.5 ± 0.1	2 ± 0.3	1.8 ± 0.2	1.9 ± 0.2	**0.006**
	D-2 (Mean ± SD)	2.9 ± 0.2	2.7 ± 0.1	2.5 ± 0.1	2.7 ± 0.3	2.4 ± 0.6	2.2 ± 0.4	2.5 ± 0.1	0.13
	D-3 (Mean ± SD)	3 ± 0.2	2.6 ± 0.1	2.3 ± 0.4	2.5 ± 0.3	2 ± 1	2.1 ± 0.6	2.4 ± 0.2	0.24
	p	**0.03**	**0.01**	0.22	0.40	0.75	0.64	**0.007**	

D-1= After infection exposure (at day 18), D-2= During treatment period (at day 25), D-3= Post treatment period (at day 30)

T_0= Control group, T_1= Onion group, T_2= Garlic group, T_3= Neem group, T_4= Tulsi group, T_5= 0.1% $CuSO_4$ group and T_6= Nystatin Sulphate group

SD= Standard Deviation

Significant variation ($p \leq 0.05$) and Highly significant variation ($p \leq 0.01$)

4.2.4 Total Erythrocyte Count (TEC)

The deviation on TEC (million/cu.mm) among the experimental groups varied significantly ($p \leq 0.05$) at infection exposure period (Table 3). The TEC count was also significantly increased at control, Onion and Nystatin Sulphate groups.

4.3 Effects of synthetic antifungals and plant extracts on leucocytes

4.3.1 Lymphocyte

The mean values of lymphocyte (%) varied significantly ($p \leq 0.01$) at infection exposure on different treated groups of the study (Table 4). The variation of lymphocyte was differed significantly ($p \leq 0.05$) in Neem and Nystatin Sulphate group.

4.3.2 Heterophil

Like lymphocytes, the average values of heterophil (%) was significantly ($p \leq 0.01$) varied at infection exposure; insignificantly ($p > 0.05$) reduced at treatment and post treatment period among the treated groups (Table 4). On other hand, in Neem and Nystatin Sulphate groups, heterophil levels differed significantly.

4.3.3 Eosinophil

In the present study, no significant difference ($p > 0.05$) was observed in eosinophil (%) among the treated groups of broiler. The average values of eosinophil ranged from 2.0 to 6.3 in different treated groups after infection exposure which was compressed insignificantly during treatment stage (Table 4).

4.3.4 Basophil

Basophil (%) level was 1-2% (Table 4) among all the treated groups across the different interval of study period and the variation was not significant ($p > 0.05$).

4.3.5 Monocyte

Monocyte (%) content was also changed significantly ($p \leq 0.01$) at the infection stages. However, in groups, significant alteration was observed in control, Onion and 0.1% $CuSO_4$ group (Table 4).

4.4 Effects on biochemical parameters

4.4.1 Effects on carbohydrate, TP and albumin

4.4.1.1 Carbohydrate (Glucose)

There was no significant (p >0.05) deviation observed on glucose (mg/dl) among the treated groups in different observational periods (Table 5). Conversely, in Garlic, Neem and Nystatin Sulphate groups, glucose level was reduced gradually and varied significantly ($p \leq 0.05$).

4.4.1.2 Total Protein (TP)

On total protein (g/l), among the treated groups or within the groups, there had no significant (p >0.05) variation all over the study (Table 5).

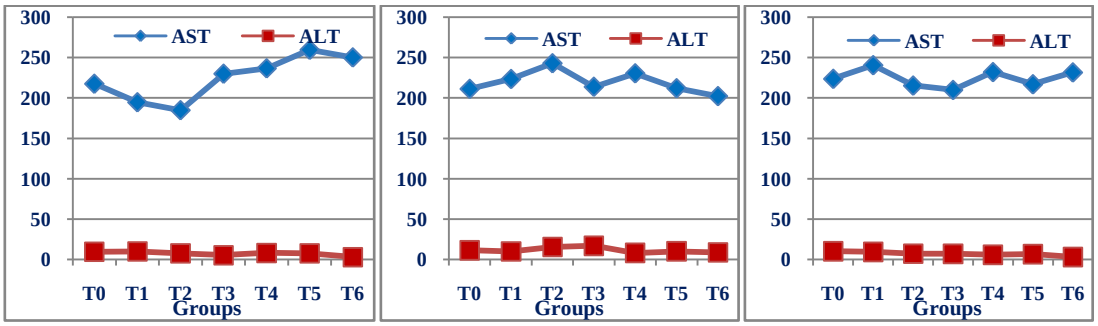
4.4.1.3 Albumin

TP was not varied significantly, but the level of albumin (g/l) was significantly ($p \leq 0.01$) varied among the treated groups at post treatment period (Table 5). Moreover, in groups, Neem group had a significant ($p \leq 0.05$) distinction comparing to other groups.

4.4.2 Effects on liver and kidney

4.4.2.1 Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT)

AST (u/l) and ALT (u/l) both are liver functional enzymes. AST and ALT both had a significant ($p \leq 0.05$) deviation after infection exposure among different treated groups where significant variation was also evident at ALT level during the treatment stage (Table 6) (Figure 24). Moreover, in Garlic and Neem groups, the variations of both enzymes were found highly significant ($p \leq 0.01$).



Period: 01 **Period: 02** **Period: 03**

Figure 24: Comparison of AST and ALT in different groups on 3 observational periods

Table 4: Effects of synthetic antifungals and plant extracts on leucocytes in broilers affected with aspergillosis

Para-meters	Periods	Groups							*p*
		T_0	T_1	T_2	T_3	T_4	T_5	T_6	
Lymph-ocytes (%)	D-1 (Mean ± SD)	70.3 ± 3.5	65.7 ± 4.6	69.3 ± 1.5	62.3 ± 2.1	72 ± 3.6	71.3 ± 1.5	63.3 ± 1.5	**0.004**
	D-2 (Mean ± SD)	66.7 ± 1.5	76 ± 7	77.7 ± 12.1	71 ± 5	68.3 ± 3.5	72 ± 4	75.3 ± 0.6	0.28
	D-3 (Mean ± SD)	64.7 ± 2.1	68.3 ± 6.7	71.3 ± 1.2	68.3 ± 3.2	70.3 ± 4.2	68.7 ± 11	69.3 ± 1.2	0.82
	p	0.08	0.19	0.38	**0.05**	0.53	0.82	**0.0000**	
Hetero-phil (%)	D-1 (Mean ± SD)	22 ± 3.6	18.3 ± 2.9	19.7 ± 0.6	29.3 ± 2.3	20.7 ± 4	18.3 ± 1.5	26.7 ± 2.9	**0.001**
	D-2 (Mean ± SD)	22.3 ± 2.1	16.3 ± 5.1	15.3 ± 8.7	19.7 ± 4	23 ± 4.4	22.3 ± 2.3	16 ± 1	0.22
	D-3 (Mean ± SD)	24.3 ± 0.6	21 ± 4.6	19.3 ± 3.2	19.3 ± 0.6	19.7 ± 1.5	24.7 ± 10.6	22.7 ± 1.2	0.61
	p	0.49	0.46	0.58	**0.006**	0.53	0.50	**0.001**	
Eosino-phil (%)	D-1 (Mean ± SD)	3.7 ± 1.5	6.3 ± 1.2	4.3 ± 3.2	3.7 ± 2.1	2 ± 0	2.3 ± 0.6	2.7 ± 1.2	0.09
	D-2 (Mean ± SD)	3 ± 1	2.7 ± 0.6	2 ± 1	2.3 ± 0.6	2.7 ± 1.2	1.7 ± 0.6	3.3 ± 2.3	0.65
	D-3 (Mean ± SD)	5 ± 2.7	3.7 ± 2.9	2.7 ± 0.6	5.3 ± 1.2	4 ± 2.7	2.7 ± 1.2	3.3 ± 1.5	0.56
	p	0.45	0.11	0.39	0.10	0.39	0.37	0.87	
Baso-phil (%)	D-1 (Mean ± SD)	1.3 ± 0.6	1 ± 1	2.3 ± 0.6	1.3 ± 1.5	1.7 ± 1.2	1.7 ± 0.6	1 ± 1	0.67
	D-2 (Mean ± SD)	1.7 ± 0.6	1 ± 1.7	1 ± 1.7	1.7 ± 0.6	1 ± 1	0.7 ± 0.6	0.3 ± 0.6	0.73
	D-3 (Mean ± SD)	1.7 ± 1.5	1.3 ± 1.2	1 ± 0	2 ± 0	1 ± 1	0.3 ± 0.6	1 ± 1	0.45
	p	0.90	0.94	0.28	0.70	0.69	0.07	0.59	
Mono-cytes (%)	D-1 (Mean ± SD)	2.7 ± 1.2	8.7 ± 1.5	4.3 ± 2.1	3.3 ± 1.2	3.7 ± 0.6	6.3 ± 0.6	6.3 ± 2.5	**0.003**
	D-2 (Mean ± SD)	6.3 ± 1.2	4 ± 2.7	4 ± 2.7	5.3 ± 0.6	5 ± 2	3.3 ± 1.2	5 ± 2.7	0.61
	D-3 (Mean ± SD)	4.3 ± 1.5	5.7 ± 0.6	5.7 ± 1.5	5 ± 2	5 ± 0	3.7 ± 0.6	3.7 ± 1.2	0.28
	p	**0.04**	**0.05**	0.62	0.24	0.36	**0.008**	0.39	

D-1= After infection exposure (at day 18), D-2= During treatment period (at day 25), D-3= Post treatment period (at day 30)
T_0= Control group, T_1= Onion group, T_2= Garlic group, T_3= Neem group, T_4= Tulsi group, T_5= 0.1% $CuSO_4$ group and T_6= Nystatin Sulphate group
SD= Standard Deviation
Significant variation ($p \leq 0.05$) and Highly significant variation ($p \leq 0.01$)

Table 5: Effects of synthetic antifungals and plant extracts on carbohydrate, TP and albumin in broilers affected with aspergillosis

Para-meters	Periods	Groups							*p*
		T_0	T_1	T_2	T_3	T_4	T_5	T_6	
Glucose (mg/dl)	D-1 (Mean ± SD)	285 ± 38.2	230 ± 19.2	226 ± 15.7	301 ± 78.5	284 ± 38	244 ± 58	245 ± 58	0.38
	D-2 (Mean ± SD)	210.5 ± 12	221.3 ± 23	232.4 ± 12	254.3 ± 26	227.3 ± 36	275.8 ± 60	244.2 ± 35	0.30
	D-3 (Mean ± SD)	222 ± 65.7	228 ± 43	209.3 ± 46	229 ± 8.3	280 ± 67.5	215 ± 50	231 ± 33	0.67
	p	0.17	0.93	**0.02**	0.25	0.36	0.46	**0.01**	
Total Protein (g/l)	D-1 (Mean ± SD)	24.3 ± 6.1	16 ± 4.4	22.3 ± 3.1	28.3 ± 4	25.7 ± 13.3	23.7 ± 1.5	30 ± 10.8	0.39
	D-2 (Mean ± SD)	26 ± 2	28.7 ± 15.1	32.7 ± 11.7	24.3 ± 6.5	28.3 ± 7.2	27 ± 8.5	22 ± 9.5	0.87
	D-3 (Mean ± SD)	27.7 ± 3.8	27.3 ± 4	27.3 ± 2.1	27 ± 3.5	28.7 ± 2.3	22.3 ± 2.1	28 ± 1.7	0.24
	p	0.66	0.27	0.28	0.61	0.90	0.55	0.52	
Albumin (g/l)	D-1 (Mean ± SD)	10.6 ± 1.7	7.5 ± 1.9	10.4 ± 0.2	12.3 ± 1.3	11.8 ± 4.8	10.8 ± 0.9	12.2 ± 3.6	0.33
	D-2 (Mean ± SD)	12.1 ± 0.7	12.5 ± 5.9	13.7 ± 4.6	9.9 ± 1.4	11.3 ± 2.2	11.9 ± 2.5	11.1 ± 3.3	0.88
	D-3 (Mean ± SD)	16.6 ± 8.7	10.7 ± 1.2	13 ± 1.4	11.2 ± 1.3	12.5 ± 0.6	11.4 ± 1.9	10.3 ± 0.9	**0.003**
	p	0.39	0.31	0.37	**0.05**	0.89	0.77	0.73	

D-1= After infection exposure (at day 18), D-2= During treatment period (at day 25), D-3= Post treatment period (at day 30)

T_0= Control group, T_1= Onion group, T_2= Garlic group, T_3= Neem group, T_4= Tulsi group, T_5= 0.1% $CuSO_4$ group and T_6= Nystatin Sulphate group

SD= Standard Deviation

Significant variation ($p \leq 0.05$) and Highly significant variation ($p \leq 0.01$)

Table 6: Effects of synthetic antifungals and plant extracts on liver and kidney in broilers affected with aspergillosis

Para-meters	Periods	Groups							*p*
		T_0	**T_1**	**T_2**	**T_3**	**T_4**	**T_5**	**T_6**	
AST (u/l)	D-1 (Mean ± SD)	217.4 ± 20	194.5 ± 3.3	184.7 ± 3.7	229.8 ± 1.3	236.5 ± 32	259.4 ± 42	250 ± 25.7	**0.01**
	D-2 (Mean ± SD)	211.2 ± 13.5	223.4 ± 10.5	242.9 ± 11	213.8 ± 1.2	230.4 ± 43.4	212 ± 15.4	202.2 ± 30.4	0.38
	D-3 (Mean ± SD)	223.6 ± 46.1	240.4 ± 38.2	215.3 ± 22.9	209.9 ± 11.1	231.9 ± 23.2	216.9 ± 31.3	231.5 ± 18.2	0.86
	p	0.88	0.12	**0.009**	**0.02**	0.97	0.21	0.14	
ALT (u/l)	D-1 (Mean ± SD)	9.7 ± 2.8	10.1 ± 2.1	7.8 ± 2.6	5.4 ± 1.7	8.3 ± 2.1	7.7 ± 1.1	3.1 ± 3.1	**0.03**
	D-2 (Mean ± SD)	11.5 ± 0.7	10 ± 2.2	15.6 ± 3.1	17 ± 3	8.2 ± 1.5	10.3 ± 5.2	8.9 ± 3.5	**0.02**
	D-3 (Mean ± SD)	10.3 ± 4.1	9.5 ± 3.9	7.3 ± 0.8	7 ± 0.7	6 ± 1.5	6.6 ± 3.5	6.8 ± 0.4	0.41
	p	0.76	0.96	**0.009**	**0.0008**	0.26	0.50	0.10	
Creati-nine (mg/dl)	D-1 (Mean ± SD)	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.32
	D-2 (Mean ± SD)	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.96
	D-3 (Mean ± SD)	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.63
	p	0.37	0.59	0.81	0.58	0.17	0.18	0.87	

D-1= After infection exposure (at day 18), D-2= During treatment period (at day 25), D-3= Post treatment period (at day 30)
T_0= Control group, T_1= Onion group, T_2= Garlic group, T_3= Neem group, T_4= Tulsi group, T_5= 0.1% $CuSO_4$ group and T_6= Nystatin Sulphate group
SD= Standard Deviation
Significant variation ($p \leq 0.05$) and Highly significant variation ($p \leq 0.01$)

4.4.2.2 Creatinine

The kidney function enzyme like creatinine (mg/dl) level was 0.4 to 0.6 (Table 6) among all the treated groups across the different interval of the study and the variation was not significant ($p > 0.05$).

4.4.3 Effects on lipid profile

4.4.3.1 Cholesterol

Insignificantly ($p > 0.05$) increased cholesterol (mg/dl) level was observed at infection exposure and post treatment period whereas reduced level was evident during the treatment episode among the treated groups (Table 7). Within the groups, significant ($p \leq 0.05$) alteration was observed in control and Neem group.

4.4.3.2 Triglycerides (TG)

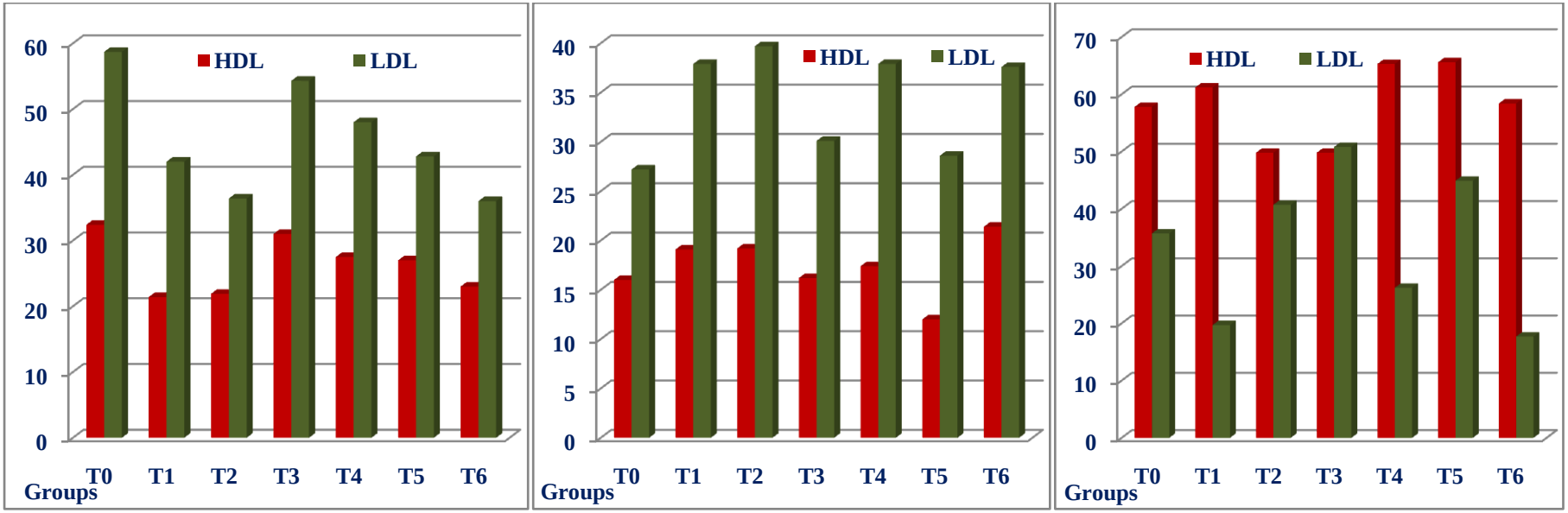
Significant ($p \leq 0.05$) variation on TG (mg/dl) was observed at treatment and post treatment period comparing to infection exposure among the treated groups (Table 7). It was also evident that, the variation on control, Tulsi, 0.1% $CuSO_4$ and Nystatin Sulphate groups were highly significant ($p \leq 0.01$) within the groups.

4.4.3.3 High Density Lipoprotein (HDL)

Reduced level of HDL (mg/dl) was observed at the infection exposure and increased after the treatment ($p > 0.05$). On the other hand, HDL varied significantly ($p \leq 0.01$) in every treated group of the study (Table 7) (Figure 25).

4.4.3.4 Low Density Lipoprotein (LDL)

The level of LDL (mg/dl) was quite opposite to the level of HDL in this study (Figure 25). LDL level was decreased after the treatment (Table 7) and the variation was significant ($p \leq 0.05$). On the other hand, LDL level was insignificantly higher during the period of infection. Moreover, in groups, LDL was significantly varied on the Neem group compared to other groups.



Period: 01 **Period: 02** **Period: 03**

Figure 25: Comparison of HDL and LDL in different groups on 3 observational phases

Table 7: Effects of synthetic antifungals and plant extracts on lipid profiles in broilers affected with aspergillosis

Para-meters	Periods	Groups							*p*
		T_0	**T_1**	**T_2**	**T_3**	**T_4**	**T_5**	**T_6**	
Chole-sterol (mg/dl)	D-1 (Mean ± SD)	137.6 ± 46.7	99.7 ± 13.4	107.5 ± 8.9	132.8 ± 25.3	111.3 ± 56.8	104.3 ± 27.4	101.3 ± 32.2	0.72
	D-2 (Mean ± SD)	69.3 ± 3.4	98.4 ± 27.8	103.6 ± 18	75.6 ± 15.9	90.3 ± 15.6	69.2 ± 22.8	89.3 ± 13.3	0.18
	D-3 (Mean ± SD)	124.3 ± 15.5	115.9 ± 6.3	138 ± 21.5	131.4 ± 13.6	112 ± 21.1	132.7 ± 27.7	103.2 ± 5.2	0.24
	p	**0.05**	0.47	0.09	**0.01**	0.72	0.06	0.68	
Tri-glycer-ides (mg/dl)	D-1 (Mean ± SD)	233 ± 57.6	181.9 ± 74	245.9 ± 0.8	237.5 ± 56	178 ± 15.3	172.4 ± 9.9	211.5 ± 10	0.21
	D-2 (Mean ± SD)	130.3 ± 11	207 ± 35.7	223.6 ± 43	146 ± 44.5	175 ± 16.1	143.1 ± 33	151.4 ± 25	**0.02**
	D-3 (Mean ± SD)	154 ± 17.4	175 ± 11	181.2 ± 43	154 ± 63.3	102 ± 11.5	111.3 ±15	135.3 ± 15	**0.05**
	p	**0.03**	0.70	0.15	0.16	**0.001**	**0.04**	**0.004**	
HDL (mg/dl	D-1 (Mean ± SD)	32.4 ± 10.2	21.4 ± 5.8	21.9 ± 3.5	31 ± 9.4	27.5 ± 22.5	27 ± 10.4	23 ± 12.8	0.87
	D-2 (Mean ± SD)	16 ± 2.1	19.1 ± 7.4	19.2 ± 5.6	16.2 ± 4.2	17.4 ± 5.5	12 ± 2.3	21.4 ± 3.5	0.34
	D-3 (Mean ± SD)	57.8 ± 17.2	61.2 ± 1.8	49.8 ± 0.8	49.8 ± 0.8	65.3 ± 19	65.6 ± 29.1	58.4 ± 6.2	0.89
	p	**0.01**	**0.0001**	**0.0008**	**0.001**	**0.03**	**0.03**	**0.003**	
LDL (mg/dl)	D-1 (Mean ± SD)	58.7 ± 29	42 ± 5.2	36.4 ± 5.7	54.3 ± 9.6	48 ± 34.4	42.8 ± 15	36 ± 21.3	0.76
	D-2 (Mean ± SD)	27.2 ± 4.2	37.9 ± 15.6	39.7 ± 5	30.1 ± 14.6	37.9 ± 9.7	28.6 ± 17.5	37.6 ± 18.6	0.82
	D-3 (Mean ± SD)	35.7 ± 9.6	19.7 ± 5.6	40.7 ± 27.8	50.8 ± 4.7	26.2 ± 10.1	44.9 ± 15.4	17.7 ± 12.1	**0.04**
	p	0.16	0.07	0.95	**0.05**	0.50	0.45	0.38	

D-1= After infection exposure (at day 18), D-2= During treatment period (at day 25), D-3= Post treatment period (at day 30)

T_0= Control group, T_1= Onion group, T_2= Garlic group, T_3= Neem group, T_4= Tulsi group, T_5= 0.1% $CuSO_4$ group and T_6= Nystatin Sulphate group

SD= Standard Deviation

Significant variation ($p \leq 0.05$) and Highly significant variation ($p \leq 0.01$)

Chapter-5: Discussion

Infection by *Aspergillus sp.* has been reported in almost all domesticated avian species and *Aspergillus fumigatus* is considered as a major respiratory pathogen. Arne et al. (2011), described that *Aspergillus fumigatus* causes the disease namely brooder pneumonia in young poultry. The number of researchers like Dhama et al. (2012), Beernaert et al. (2010), Martin et al. (2007), Kunkle (2003), Akan et al. (2002) and Joseph (2000) isolated other species of moulds such as *A. flavus*, *A. niger*, *A. nidulans* and *A. terreus* from avian aspergillosis.

In the current study, infected broilers were suffered in gasping, suffocation, dyspnoea, coughing and mucus through nostrils and the observation was found similar to that previously identified in different birds by Dhama et al. (2011), Cacciuttolo et al. (2009), Nardoni et al. (2006), Atasever and Gumussoy (2004), Jones and Orosz (2000).

Yellow colored uniform pin point lesions were visible on the lungs, which ranged from miliary to larger granulomatous foci as "Sagu" appearance. On the other hand, walls of air sacs were thickened and bronchioles filled with mucus and congested lungs were found. These lesions are similar to the post mortem findings of aspergillosis affected birds described elsewhere (Cacciuttolo et al., 2009; Nardoni et al., 2006; Shivaprasad, 2000; Calnek et al., 1997).

5.1 Efficacy of synthetic drugs and medicinal plant extracts as antifungal

Nystatin sulphate which is an active agent against mucocutaneous candidiasis exhibited lesser efficacy against aspergillosis in this study. Treating litter combinedly with Nystatin sulphate and 0.1% $CuSO_4$ can reduce mold content in litter (Dyar et al., 1984). In the study, 0.1% $CuSO_4$ used singly in water against aspergillosis, this is in agreement with Johnson and Fiske (1935).

This *in vivo* experimental study observed antifungal efficacy of all studied plants (Nem, Tulsi, Garlic and Onion) which was evaluated earlier by Bansod and Rai (2008) where they found *in vitro* antifungal efficacy.

Ethanol extracted Neem leaf had a greater inhibitory effect on *Aspergillus spp.* which supported *in vitro* study of Margaret et al. (2013), Mahmoud et al. (2011) and Mondali et al. (2009) and differed from Rai et al. (2013). The fungicidal and bactericidal properties of extracted Neem leaf contain several antimicrobial active ingredients such as desactylimbin, quercetin and sitosterol either *in vitro* or *in vivo* trials (Singh et al., 1980) whereas other researchers (Ghorbanian et al., 2007; Bhatnagar, 1988) explained this activity by the presence of active ingredients like triterpenes or the limonoids such as meliantriol, azadirachtin, desactylimpin, quercetin, sitosterol, nimbin, nimbinin, nimbidin, nimbosterol and margisine.

This study showed that the ethanolic extract of Tulsi leaf has lesser extent of fungicidal activity. Aqueous leaf extract of 40% concentration recorded as effective for *A. niger* while inhibition was increased with higher concentration of plant extract (Gupta et al., 2014). According to Sharma (2010), increased concentration of leaf extracts also showed inhibitory effects against *A. fumigatus*.

The results of present study revealed that Garlic had better antifungal efficacy than other three plants and synthetic antifungals, in consistent with the findings of Ismaiel et al. (2012) where he observed effective antifungal activity in rabbits. The action of Garlic is manifold, because of fungistatic substances, allicin and other sulfur compounds. The antibacterial and antifungal properties of Garlic have been described in various *in vitro* or *in vivo* studies (An et al., 2009; Ogita et al., 2009; Tedeschi et al., 2007).

However, like Garlic, another *Allium sp.*, Onion showed antifungal properties both *in vivo* and *in vitro* studies. Onion oil contains allicepin (Wang and Ng, 2004) which reduces fungal growth and inhibits aflatoxin production by *Aspergillus flavus*, *Aspergillus parasiticus* var. *globosus*, *Aspergillus versicolor* (Zohri et al., 1995).

5.2 Effects of synthetic antifungals and medicinal plants on growth performance

Significantly increased body weight and FCR were observed on both Nystatin Sulphate and 0.1% $CuSO_4$ treated birds which were supported by Johnson and Fiske

(1935) and differed with Jegede et al. (2011) in broilers on $CuSO_4$ diets. Moreover, an improved FCR on $CuSO_4$ fed at the end of Day-21 and Day-42 was recorded by Samanta et al. (2011).

Better performance on the final body weight was recorded in Neem treated group which agreed with Rahman et al. (2014), Ansari et al. (2008), Manwar et al. (2005) and contrary to Nayaka et al. (2013), Kumar and D'Mello (1995). In this group, weekly weight gain was similar to control group which is in agreement with Sarker et al. (2014), Onyimonyi et al. (2009), Verma et al. (1998), Chakravarty and Prasad (1991). Neem treated birds had negative effect on FCR as compared to control and other treated groups. The findings were in agreement with Nayaka et al. (2013), Esonu et al. (2006), Kumar and D'Mello (1995) and differing to Rahman et al. (2014), Nagalakshmi et al. (1996). However, no marked variations in FCR of birds fed Neem leaf were reported by earlier researchers (Kabeh and Jalingo, 2007).

Inconsistent growth performance was recorded in broilers treated with ethanol extracted Tulsi and this observation is supported by Varaprasad Reddy et al. (2007) and conflicting with Biswas (2013), Sanjyal and Sapkota (2011), Lanjewar et al. (2008), Funde (2005) where they concluded as improved production performance and final live weight in broilers. As like as Neem, birds showed negative effect on FCR following the use of Tulsi which is similar to Lanjewar et al. (2008) and opposing with Nath et al. (2012), Sanjyal and Sapkota (2011), Joshi et al. (2009). Decreasing trends of weight gain was observed which is similar to Sanjyal and Sapkota (2011).

Improved growth performance with a significantly positive relationship between supplementation of Garlic extract and final body weight, weekly body weight gain and FCR was also evident in Garlic treated group. These findings are in the agreement with Onyimonyi et al. (2012), Vidica et al. (2011), Dieumou et al. (2009), Bampidis et al. (2005), Lewis et al. (2003), Freitas et al. (2001) and differed by Issa and Omar (2012), Raeesi et al. (2010), Demir et al. (2003), Chowdhury and Smith (2002), Konjufca et al. (1997). Highest body mass was recorded as 1354g ± 76 at the end of fourth week which is supported by Horton et al. (1991).

Moreover, better FCR (1.66) was recorded in Garlic group. But according to Raessi et al. (2010), supplementation of 1% Garlic extract decreased FCR compared with 0.5% Garlic. Lewis et al. (2003) reported improved FCR in broilers between 7-27 days where Alcicek et al. (2003) and Demir et al. (2003) found lower FCR between 18-28 days. Birds received Garlic, had higher feed consumption with lower FCR which is in agreement with Raeesi et al. (2010). Though Garlic produces intense pungent smell in feed and chickens required a period to adapt which is not continued in this study and conflicting to Hernandez et al. (2004).

Consistent increased growth performance was observed in the broilers treated with Onion and this study explores positive association between Onion extract and the final body weight. According to Kook et al. (2009), body weight was differed significantly between the control and Onion treated group. Moreover, slightly higher FCR with a higher feed consumption was recorded by Kook et al. (2009).

5.3 Effects of synthetic antifungals and plant extracts on hematology

The mean values of ESR, PCV, Hb and TEC in Neem treated group was not differed from the control and other treated groups which is in agreement with Rahman et al. (2014), Nnenna and Okey (2013), Aruwayo et al. (2011) and opposed by Nayaka et al. (2013), Biu et al. (2009), Gowda et al. (1998).

Moreover, significantly increased lymphocytes and lowered heterophil with slight elevation of monocytes were revealed on Neem treated group which is similar to Esonu et al. (2006) and varied from Nayaka et al. (2013). Gangar et al. (2006) and Borjesson et al. (2000) reported higher monocyte count in Neem treated group.

In Tulsi treated group, the mean values of ESR, PCV, Hb and TEC was not varied compared to control and other treated groups which is agreed by Nath et al. (2012) and differed by Biswas (2013).

Reduction of Hb concentration was observed where monocyte (%) was varied during 0.1% $CuSO_4$ treatment which opposed the findings of Jegede et al. (2011) in broilers on Copper supplementation. On other hand, significant variation in TEC count was

observed while raise of lymphocytes and reduction of heterophil was realistic due to infection.

5.4 Effects of synthetic antifungals and plant extracts on serum biochemistry

On Neem treated group, significant reduction of AST was observed while variation was found in ALT which is completely opposed by Aruwayo et al. (2011) and Musalia et al. (2000), reporting insignificant alteration in ALT and significant deviation in AST. On other hand, Dafalla et al. (2012) observed significant increase in ALT level of albino rats using higher doses of Neem extract and it is due to development of hepetonephropathy in liver (Razzaghi-Abyaney et al., 2005). According to Aruwayo et al. (2011), Musalia et al. (2000) and Jacobson (1995), Neem causes mild to severe changes in kidney, liver, spleen, intestine and heart of chicks which is not support by this study due to no variation in creatinine level. Significant variation on albumin and insignificant reduction of glucose was also evident in Neem treated group which is contradictory to the findings of Nnenna and Okey (2013) and Halim (2003).

Moreover, during Neem treatment, significant elevation of lipid metabolites (cholesterol, HDL, LDL) indicated fat deposition in broilers which is not an agreement with Nnenna and Okey (2013), Ogbuewu et al. (2010a, b), Uko and Kamalu (2008), Chattopadhyay et al. (1993) where they showed significant reduction of serum cholesterol without changing serum protein, blood urea, uric acid and suggested a general decrease in lipid metabolism. Ketkar (1976) observed higher unsaturated fatty acids in Neem than saturated fatty acids and the degree of fatty acid saturation is directly related to the amount of blood lipids (Zulet et al., 1999; Katan et al., 1994) consequently to fat deposition. On other hand, reduction in TG with neem extract had also been reported and lower levels of TG may be due to the inhibition of fatty acids synthesis (Pesti, 1997).

Elevation of HDL and reduction of TG in Tulsi treated group were observed in experimental broiler birds but insignificant alterations were analyzed on ALT, AST and creatinine which is agreed by Gupta and Charan (2007). Supplementation of

broiler diets with Tulsi extract had insignificant cholesterol concentration, whereas a significant increase in HDL (Deshpande, 2006) is coincided with this study.

Significant rise of HDL was also explored in broilers while treated with Onion extract. This finding is supported by Yanagita et al. (2003) and opposed by Kumari and Augusti (2007) where concluding as reduced lipid metabolites in Sprague-Dawley rats. On the other hand, variation in cholesterol, HDL and TG were observed between the untreated and treated group in Onion supplement studies (Kook et al., 2009). Moreover, Yanagita et al. (2003) did not found significant effect on ALT, AST through dietary Onion which is coincided with the study.

Garlic as treatment caused a significant rise in blood HDL on lipid metabolites which is completely agreed with Issa and Omar (2012), Rahimi et al. (2011) and Prasad et al. (2009) where they also recorded reduction of cholesterol, LDL and TG in broiler chicken during feeding with Garlic supplement. These hypocholesterolemic effects on chickens were occurred through inhibition of cholesterol and lipids synthesis and also the synthesis of fatty acids (Stanacev et al., 2011; Botsoglou et al., 2004). Moreover, Eidi et al. (2006) reported that Garlic extract decreased total cholesterol, triglycerides in diabetic rats.

Reductions in triglyceride and an elevated concentration of HDL were observed in broilers while treated with both Nystatin Sulphate and 0.1% $CuSO_4$. The findings agree with previous reports of Jegede et al. (2011) and Samanta et al. (2011) where dietary supplement of $CuSO_4$ was also found to cause a significant decrease in cholesterol. On contrary, serum glucose level was also significantly reduced in Nystatin Sulphate treatment.

Chapter-6: Conclusion

In this study, it was observed that Garlic and Onion bulb extract treated groups had improved growth performance than ethanol extracted Neem and Tulsi. In Tulsi treated group, growth performance was inconsistent and lipid metabolites TG and HDL were significantly altered, and liver function enzymes both AST and ALT were varied in both Garlic and Neem. No hematological alteration was present in Garlic but increased lymphocyte and reduced heterophil was observed both in Neem and synthetic Nystatin Sulphate group. So it can be concluded that, Garlic had better antifungal efficacy which is followed by Onion, Nystatin Sulphate and 0.1% $CuSO_4$. Though Neem had good efficacy but it altered almost all biochemical parameters wherever Tulsi had lesser antifungal efficacy.

Chapter-7: Recommendations and Future Perspective

Antifungal efficacy was observed in both ethanol extracted Neem and Tulsi leaves as well as bulb of Garlic and Onion. On the basis of this experiment following recommendations are given-

1. Specific concentrations of each leaf or bulb extract should be determined *in vitro* which will be more effective *in vivo* against the disease
2. Exact active ingredients which act as antifungal of ethanol extracted leaves of Tulsi, Neem and Onion, Garlic bulb extract must be identified by using Gas Chromatography Mass Sphectometry (GCMS) or Nuclear Magnetic Resonance (NMR) or Infra-red (IR) technique
3. Toxicity studies of the effectual plants should be done to establish the safety indices of the extract
4. Antifungal residue in carcass will be explored by using Thin Layer Chromatography (TLC) or UHPLC (Ultra-High Performance Liquid Chromatography) for withdrawal period determination
5. Carcass quality of treated birds would be evaluated by Association of Official Analytical Chemist (AOAC) methods which will be safe for consumers
6. Need to find out the mechanisms of the action, compatibility with other drugs, such as Amphotericin B, Itraconazole etc side effects and other important parameters
7. Future investigation is required for combined dosages of Garlic and Onion extract to provide a better therapy in the management of *Aspergillus* infection
8. Plants should be further studied to investigate the appropriate antifungal efficacy on *Aspergillus spp.* as well as diversified fungal infection and also for antibacterial or anthelmintic or anticytotoxic properties.

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Appendix-A

Preparation of ethanol extract of Neem and Tulsi

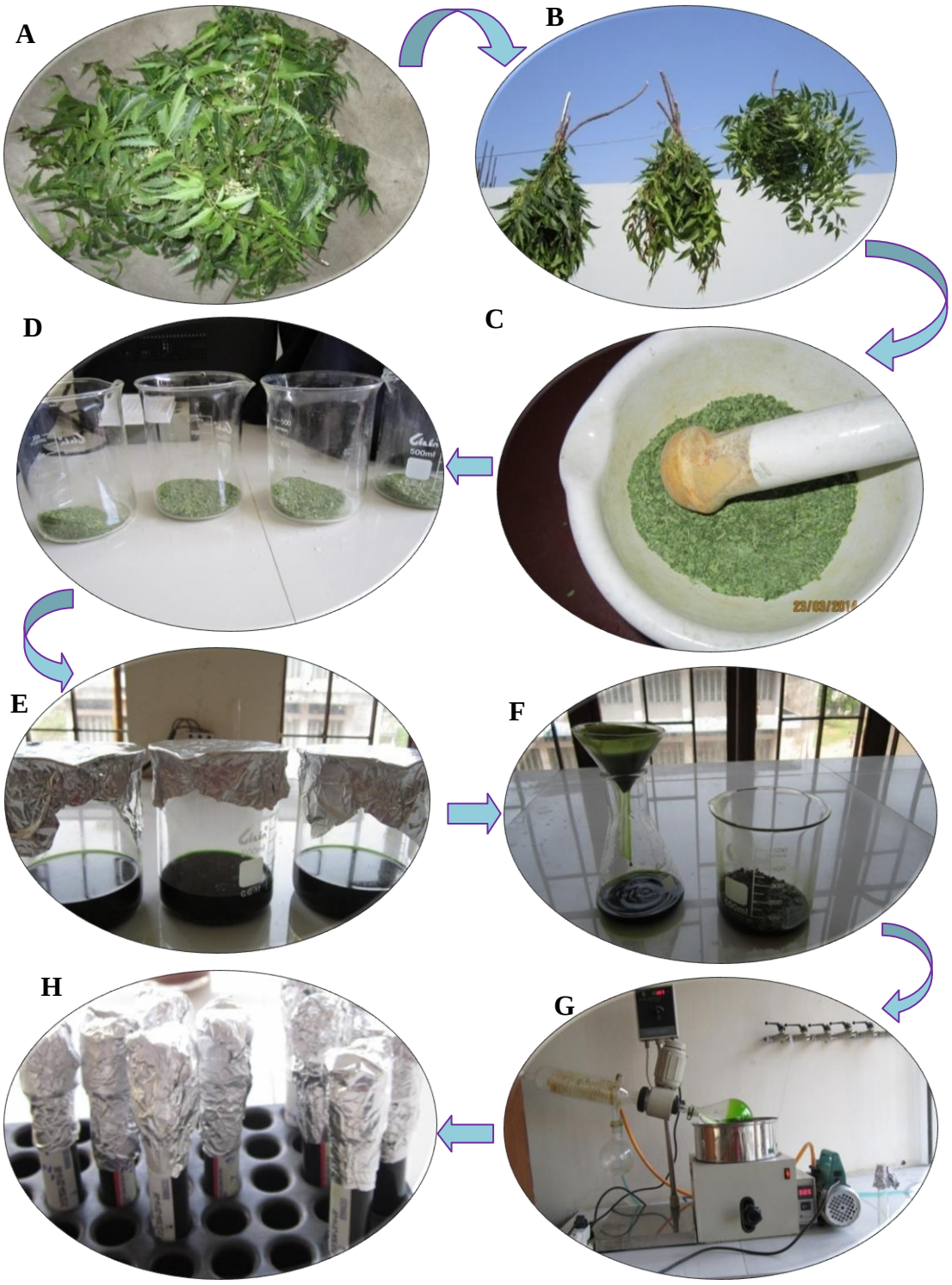

Figure 26: A. Collection of leaves, B. Sun drying (Oven also used), C. Grinding, D. Dust in beaker, E. Dust mixed with ethanol, F. Mixture filtration, G. Evaporation of solvent, H. Preserved in capped tubes.

Appendix-B

Table 8: Ingredients and Nutritive composition of the experimental broiler grower diets

Ingredients	Grower ration (14-28 days) (Kg/100Kg)
Maize	58.0
Auto Rice Polish	3.75
Soybean oil	3.2
Molasses	0.6
Soybean Meal	27.5
Protein Concentrate	3.0
Meat and Bone meal	2.0
Limestone	1.0
DCP	0.5
Salt	0.25
Vitamin mineral premix	0.25
Cholin Chloride	0.10
L-lysin	0.05
DL-Methionine	0.10
Antioxidant	0.0125
Total	100.3125
Estimated chemical composition (DM basis)	
Metabolizable Energy (Kcal/kg)	3080
Crude Protein (g/100g)	21.06
Crude Fiber (g/100g)	3.68
Calcium (g/100g)	0.95
Phosphorous (g/100g)	0.74
Lysin (g/100g)	1.29
DL Methionine (g/100g)	0.52

Appendix-C

Histopathological study

For Histopathological study formalin fixed tissue samples were washed and dehydrated in graded ethanol and embedded in paraffin wax. Fixed tissues were sectioned at 5µm thickness and stained with Periodic Acid Schiffners (PAS) as per standard method.

Collection of samples and processing

During tissue collection the following point were taken into consideration; the tissues were collected in conditions as fresh as possible. Normal and diseased tissues were collected side by side. The thickness of the tissues were as less as possible (5mm approximately). Formalin fixed tissues were processed by following protocol.

Fixation: 10% neutral buffered formalin was added in the plastic container. (10 folds of the tissue size and weight) and fixed for 3-5 days.

Washing: The tissues were trimmed into a thin section and washed over night in running tape water to remove formalin.

Dehydration: The tissues were dehydrated by ascending ethanol series to prevent shrinkage of cells as per following schedule. The tissues were dehydrated in 50%, 70%, 80%, 95%, 100%, 100% ethanol one hour in each.

Cleaning: The tissues were cleaned in chloroform for 3 hours to remove ethanol (two changes; one and half hr in each).

Impregnation: Impregnation was done in melted paraffin (56-60°C) for 3hr.

Sectioning: Then the tissues were sectioned with a microtome at 5µm thickness. A small amount of gelatin was added to the water bath for better adhesion of the section to the slide. The sections were allowed to spread on warm water bath at 40-42°C. Then the sections were taken on grease free clear slides.

Drying: The slides containing section were air dried and kept in cool place until staining**.**

Reagents:

1. Absolute alcohol (100% ethanol)
2. Glacial Acetic Acid
3. Amylase
4. Chloroform
5. Periodic Acid
6. Permount
7. Reagent alcohol
8. Schiff Reagent
9. Xylenes

Solutions:

1. Carnoy's Fixative
   Alcohol, 100% 60ml
   Chloroform 30ml
   Glacial acetic acid 10ml
2. Periodic Acid Solution, 0.5% (w/v): Periodic acid 50mg dissolved in deionized water 1 ml
3. Alcohol 50 %, 70%, 80%, 95%
   Reagent alcohol ~50ml, 70ml, 80ml, 95ml respectively
   Deionized water ~50ml, 30ml, 20ml, 5ml accordingly

Staining Procedure:

1. Place the cover slip with section in a Columbia staining dish.
2. Add Carnoy's fixative to dish for 10 minutes.
3. Rinse very carefully with several exchanges of deionized water.
4. Add Periodic Acid solution to staining dish for 10 minutes.
5. Rinse very carefully with several exchanges of deionized water.
6. Add Shiff Reagent for 5 minutes.
7. Carefully wash with three exchanges of tap or deionized H_2O.
8. Dehydrate in ascending alcohol solutions (50%, 70%, 80%, 95% X 2 and 100% X 2) in Columbia staining dishs (jars).
9. Clear with xylene (3-4X) also in Columbia staining dish (jar).
10. Mount cover slip onto a labeled glass slide with per mount or some other suitable organic mounting medium.

Appendix-D

Serum Biochemical Assays

Glucose assay

Assay principle

The principles outcome of glucose is based on the principle of competitive binding between glucose in the test specimen and GOD-PAP reagent of glucose. The glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The formed hydrogen peroxide reacts under catalysis of peroxidase with phenol and 4-aminophenazone to a red-violet quinoneimine dye as indicator.

Reaction

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneinine} + 4\ H_2O$$

Materials and reagents

1. Serum sample
2. Glucose conjugate reagent
3. Precision pipettes 10µl, 1.0ml
4. Eppendorf tube, eppendorf tube holder, disposable pipette tips, distilled water, 70% alcohol, absorbent paper or paper towel or cotton and gloves.

Procedure

The sterile eppendorf tubes were taken. 1000µl of Glucose conjugate reagent was taken each into each eppendorf tube. Then 10µl of Glucose standard was added in with the reagent in eppendorf tube and 10µl of samples serum were taken in each sample eppendorf tube. The eppendorf tube was then incubated at 37ºC for 10 minutes. Glucose standards with conjugate reagent were examined first to determine the standard value. Then all eppendorf tubes containing sample serum with Glucose conjugate reagent was examined by Biochemical analyzer and the reading was taken. The standard value was used as a compared tool.

Total protein assay

Assay principle

The principle outcome of total protein is based on the principle of competitive bindings between cupric ions react with protein in alkaline solution to form a purple complex. The absorbance of this complex is proportional to the protein concentration in the sample.

Materials and reagents

1. Serum sample
2. Total protein conjugate reagent

3. Precision pipettes: 20µl and 1.0ml
4. Eppendorf tube, eppendorf tube holder, disposable pipette tips, distilled water, 70% alcohol, absorbent paper or paper towel or cotton and gloves.

Procedure

This was a photometric colorimetric test for total proteins, called Biuret method. The sterile eppendorf tubes were taken. Then 20µl of total protein standards was taken in an eppendorf tube and 20µl of sample serums were taken in each 24 eppendorf tube. 1000µl of total protein conjugate reagent was then added to each eppendorf tube. The eppendorf tube was then incubated at 37ºC for 10 minutes. Total protein standards with conjugate.

Albumin assay

Assay principle

The principles outcome of albumin is based on the principle of competitive bindings between albumin and albumin reagent. Bromocresol green forms with albumin in citrate buffer a colored complex. The absorbance of this complex is proportional to the albumin concentration in the sample.

Materials and reagents

1. Serum sample
2. Albumin conjugate reagent
3. Precision pipettes
4. Eppendorf tube, eppendorf tube holder, disposable pipette tips, distilled water, 70% alcohol, absorbent paper or paper towel or cotton and gloves.

Procedure

This was a photometric colorimetric test for albumin is called Bromo Cresol Green method. The sterile eppendorf tubes were taken. Then 10µl of albumin standards was taken in an eppendorf tube and 10µl of sample sera were taken in each eppendorf tube. 1000µl of albumin conjugate reagent was then added to each eppendorf tube. The eppendorf tube was then incubated at 37ºC for 5 minutes. Albumin standards with conjugate reagent were examined first for determined of the standard value. Then all 100 eppendorf tubes containing sample serum with albumin conjugate reagent was examined using automated humalyzer and the reading was taken. The standard value was used as a compared tool.

Cholesterol assay

Assay principle

The principles outcome of cholesterol is based on the principle of competitive bindings between cholesterol and cholesterol reagent. The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed hydrogen peroxide and 4-aminophenazone in the presence of phenol and peroxidase.

The absorbance of this complex is proportional to the cholesterol concentration in the sample.

Reaction

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterage}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesterol-3-one} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{quinoneimine} + 4H2O$$

Materials and reagents

1. Serum sample
2. Cholesterol conjugate reagent
3. Precision pipettes
4. Eppendorf tube, eppendorf tube holder, disposable pipette tips, distilled water, 70% alcohol, absorbent paper or paper towel or cotton and gloves.

Procedure

This was an enzmatic colorimetric test for cholesterol, called CHOD-PAP method. The sterile eppendorf tube was taken. Then 10µl of cholesterol standards was taken in an eppendorf tube and 10µl of sample serums were taken in each eppendorf tube. 1000µl of cholesterol conjugate reagent was then added to each eppendorf tube. The eppendorf tube was then incubated at 37ºC for 10 minutes. Cholesterol standards with conjugate reagent were examined first for determined of the standard value. Then all eppendorf tubes containing sample serum with cholesterol conjugate reagent was examined by automated humalyzer and the reading was taken. The standard value was used as a compared tool.

Triglyceride

Assay Principle

The triglycerides were determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4 – aminophenezone and 4 – chlorophenol under the catalytic influences of peroxidease.

Materials and reagent

1. Serum sample
2. TG conjugate reagent
3. Precision pipettes
4. Eppendorf tube, eppendorf tube holder, disposable pipette tips, distilled water, 70% alcohol, absorbent paper or paper towel or cotton and gloves

Procedure

The sterile eppendorf tubes were taken. Then 1000µl TG standards was taken in an eppendorf tube and 10µl of sample serums were taken in each eppendorf tube The eppendorf tube was then kept in room temperature for 10 minute. TG standards with conjugate reagent were examined first for determined of the standard value. Then all eppendorf tubes containing sample serum reagent was examined by automated humalyzer and the reading was taken. The standard value was used as a compared tool.

LDL assay

Assay Principle

The principles outcome of LDL is based on the principle of competitive bindings between LDL and LDL reagent. Low density lipoproteins are precipitated by the addition of heparin at their isoelectric point (PH-5.04). The HDL and VLDL remain in the supernatant and can be determined by enzymatic methods.

LDL Cholesterol = Total Cholesterol – Cholesterol in the supernatant. The absorbance of this complex is proportional to the LDL concentration in the sample.

Materials and reagents

1. Serum sample
2. LDL conjugate reagent
3. Precision pipettes
4. Eppendorf tube, eppendorf tube holder, disposable pipette tips, distilled water, 70% alcohol, absorbent paper or paper towel or cotton and gloves.

Procedure

The sterile eppendorf tubes were taken. Then 100µl of LDL standards was taken in an eppendorf tube and 100µl of sample serums were taken in each eppendorf tube. 1000µl of LDL conjugate reagent was then added to each eppendorf tube. The eppendorf tube was then kept in room temperature for 10 minutes and then centrifuged at 4000rpm for 15 minutes. The LDL concentration of the supernatant was determined within 1 hour after centrifugation. LDL standards with conjugate reagent were examined first for determined of the standard value. Then all eppendorf tubes containing sample serum with LDL conjugate reagent was examined by automated humalyzer and the reading was taken. The standard value was used as a compared tool.

HDL assay

Assay Principle

Low density lipoprotein (LDL and VLDL) and chylomicron fractions are precipitated quantitavily by the addition of phosphotangstic acid in the presence of magnesium ions.

After centrifugation, the cholesterol concentration in the HDL (high density Lipoprotein) fraction, which remains in the supernatant, is determined.

Materials and reagents

1. Serum sample
2. HDL conjugate reagent
3. Precision pipettes
4. Eppendorf tube, eppendorf tube holder, disposable pipette tips, distilled water, 70% alcohol.

Procedure

The sterile eppendorf tubes were taken. Then 400µl of HDL standards was taken in an eppendorf tube and 200µl of sample serums were taken in each eppendorf tube. 100µl of distilled water was then added to each eppendorf tube. The eppendorf tube was kept in room temperature for 10 minutes and then centrifuged at 4000rpm for 15 minutes. Then 50µl HDL concentration of the supernatant was taken and 1000µl Cholesterol reagent added determined within 1hr after centrifugation. HDL standards with conjugate reagent were examined first for determined of the standard value. Then all eppendorf tubes containing serum with HDL conjugate reagent was examined by automated humalyzer and the reading was taken. The standard value was used as a compared tool, absorbent paper or paper towel or cotton and gloves.

AST (Aspartate Aminotransferase) or SGOT

Procedure:

Aspirate fresh ddH_2O and perform a new Gain Calibration in flow cell mode. Select AST in the Run Test screen and carry out water blank as instructed.

Pipette into a test tube:	
Sample	0.05ml
Reagent	0.5ml

Mix and aspirate into the Rx Monza.

Pipette into cuvette:		
	Macro	Micro
Sample	0.2ml	0.1ml
R_1 Enzyme/ Coenzyme/ α-oxoglutarate	2.0ml	1.0ml

Mix, read initial absorbance after 1 min. Read again 1, 2 and 3 minutes. Note if the absorbance change per minute is between

0.11 and 0.16 at 340/Hg 334nm

0.06 and 0.08 at Hg 365nm

Use only the values for the first 2 minutes for the calculation.

ALT (Alanine Aminotransferase) or SGPT

Procedure:

Aspirate fresh ddH_2O and perform a new Gain Calibration in flow cell mode. Select ALT in the Run Test screen and carry out water blank as instructed.

Pipette into a test tube:	
Sample	0.05ml
Reagent	0.5ml

Mix and aspirate into the Rx Monza.

Pipette into cuvette:		
	Macro	Micro
Sample	0.2ml	0.1ml
R_1 Enzyme/ Coenzyme/ α-oxoglutarate	2.0ml	1.0ml

Mix, read initial absorbance after 1 min. Read again 1, 2 and 3 minutes. Note if the absorbance change per minute is between

0.11 and 0.16 at 340/Hg 334nm

0.06 and 0.08 at Hg 365nm

Use only the values for the first 2 minutes for the calculation.

Reference: Randox Laboratories Limited, 55 Diamond Road, Crumlin, Country Antrim, BT29 4QY, United Kingdom. www.randox.com

Brief Biography

Mohammed Ashif Imtiaz Shawn passed Secondary School Certificate (SSC) examination from Chittagong Collegiate School in 2003 and then Higher Secondary Certificate (HSC) examination from Chittagong College in 2005. He obtained his Doctors of Veterinary Medicine (DVM) Degree in 2013 from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. Now, he is a Candidate for the degree of MS in Pharmacology under the Department of Physiology, Biochemistry and Pharmacology, Faculty of Veterinary Medicine, CVASU. He has published four scientific articles: one in national and three in international journals. He has immense interest to work in new drug discovery in veterinary field.