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IN-VITRO EVALUATION OF PHYTASE AND NEEM IN CHICKEN INTESTINAL EPITHELIAL CELLS

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Abstract - **Back ground and objective - Phosphorus is presently discovered as a source of chicken diets in plant seeds. Phytic acid involves phosphorus, which is poorly digested, reduces use, and reduces metabolism and growth of chicken. Use of phytase and neem leaf extracts to enhance the use of phosphorus. Methodology: To prepare the neem leaves extract and commercial phytase used in the intestinal embryo chicken cell line to determine gene and enzyme activity using respectively molecular and biochemical characteristics. Trizol reagent used for RNA isolation, RNA assessment, cDNA synthesis, primary design in cellular regulatory genes, PCR amplification and RT-PCR sequences for molecular characterization. In biochemical characterization, we used protein lysis buffer to isolate protein, estimate protein by Lowry method, and assess the activity of the enzyme through alkaline phosphatase kit. The results were analyzed by two-way ANOVA test. Results: The findings of RT-PCR showed that the less time it took to amplify cDNA produces a high level of gene expression product in ALP, G-6-P, Hexa kinase, p19 and vice versa in p53, p21 cellular regulatory genes. The activity of the enzyme also showed an increase. The findings of the two-way ANOVA experiment showed that gene expression products were statistically significant ($p<0.005$) in each cellular regulatory gene in the control and therapy group. Conclusion: Involvement of Cell Cycle Regulatory and Phosphate Metabolism Phytase and Neem Extract in Chickens ' diet can be useful for enhanced nutritional effectiveness, improved use, digestion of feed and growth of broilers as a direct hydrolytic result for phytate.**

Key words - phytase, broiler chicken cell line, Neem leaves extract, phosphorous, and Gene expression

I. INTRODUCTION

Chicken is a group of domesticated birds for meat, eggs, and feathers kept by humans. Chicken manufacturing can lead to strategic revenue generation, enhancing indirect food safety pathways and mitigating disaster risk[Alders, R.Getal., 2009; Wong,J.Tetal.,2017], and Achieving without adverse effects on the, environment by combining manufacturing technologies with local agroecological technologies, using local and innovative feed formulations and ensuring that poultryfed food of human quality is used to create high dietary value secure poultry products[Kryger, K.Netal., 2010]. Ensure that all poultry products (meat and eggs) suitable for human consumption are used efficiently through surveillance of use along human and animal value chains[Wang Z, et al.,2009].These birds make a contribution without delay to food security through domestic intake and circuitously through the sale of birds to help the achievement of other food objects, progressed family healthy situations and medicines [Wong, J. T et al.; 2017].

Phosphorus takes a vital character in the broilers metabolic and development procedures in the chicken's size and a nutrient that has the third uppermost profitable rate in broiler nourishment preparation next energy and amino acids, and its usage must be raised[Woyengo T A and Nyachoti M ,(2013); M LamidC et al.,2018].

Phytases are phosphatases that can hydrolyze one or more groups of phosphate from the phytate molecule [Tamim, N. M., 2004]. The phosphate released will be used as a source of phosphorus mineral deposits for livestock [Morse D H H and Wilcox D J, 1992], and the advantageous result of exogenous phytase in poultry nutrition is the complete hydrolytic outcome on the rise in the accessibility of phytate and minerals, amino acids, and energy [Selle P H and Ravindran V,

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2007].Broiler diets consist basically of plant-derived ingredients. These feeds are characterized by having a large part of phosphorus in the form of phytate [Falowo, A. B. (2015)], which is poorly hydrolyzed by monogastric animals [Tizziani, T et al.,2016], phytase enhances food digestibility, minimizing the anti-nutritional effects and promoting the productivity indices [HOOGE, D.M et al.,2010].

Exogenous phytase is included in feed formulations not only to reduce phosphorus supplementation, but also to release minerals, particularly calcium, as well as amino acids and carbohydrates by the hydrolysis of phytate, improving nutrient utilization [Oluyinka AO et al., 2008; Slominski BA et al., 2011].Plant materials are the major constituents of poultry diets. The ability of poultry and pigs to use phytate P is poor [Ravindran, V et al,.2001; NRC, 1994; Ahmed, F et al., 2004],to insufficient quantities or lack of intestinal phytase secretion. This is due to lack of phytase, the enzyme that hydrolyses phytic acid into inositol and orthophosphate [Singh, P.K et al., 2008; Kaya, M et al., 2009].Increasing of chicken's performance using supplemented exogenous enzymes with wheat and barley based diets is correlated to higher digestion and absorption rate of nutrients through the gut [Brenes, A et al., 1993a; Brenes, A et al., 1993b; Slominski BA et al., 2011; Kalantar, M et al., 2016] and has been related to increased gene expression of nutrient transporters such as glucose, amino acids and peptides [Gilbert, E. R et al., 2008a; Gilbert, E. R et al., 2008b; Agyekum, A.K et al., 2015]. Phytic acid acts as an anti-nutritional factor due to binding with starch, proteins and minerals, such as P, Zn, Fe, Ca and Mg [Yang, Y.Y et al ., 2017]. Supplementation of phytase improves the nutritive value of feedstuffs by neutralization the negative effects such as intestinal villi atrophy, enlarged digestion organs and increased size of gastro intestinal tract [Cowieson, A. J et al., 2004; Ravindran, V et al., 2001; Kalantar, M et al., 2016].

Neem (Azadirachta indica) which is commonly called 'Indian Lilac' or 'Margosa', belongs to the family Meliaceae, subfamily Meloideae and tribe Melieae A. indica is a fast growing evergreen tree which has a potential to provide medicinal and nutritive value to broilers [Schmutterer H, (1990)] . Broilers given neem leaf extract in water show progressed nutrient verbal exchange performance and weight gain [Mahejabin, N et al., 2015)].

Therefore, the main objective of our research paper to prepared the neem leaf extract for chicken diet and purchased commercial phytase , to evaluate the growth pattern of treated and untreated groups, evaluate genome profiling and the phosphorylation path way activities in broiler chicken cell line by molecular and biochemical characterization to determine the gene and enzyme activity respectively. Now that the gene expression and enzymatic activity with the sample of phytase and neem extract done in vitro chicken cell line successfully achieved, in the future recommend will be practice in vivo methods for the demonstration of all regulatory genes it should be possible to obtain further insights in the function of phosphorylation pathway in the metabolism of cells.

II. MATERIALS AND METHODS

Materials: This study used the fresh Neem Leaves Powder prepared, Extract and commercial phytase enzyme, Phytic Acid, Nutritional medium, Intestinal Embryo Chicken Cell Line, different lab reagent and equipment.

2.1 Preparation of Neem Leaves extract
2.2 Sample Collection and solvent extract

Leaves were gathered from nearby greater Noida knowledge park lll, Sharda university Azadirachta indica tree and safely taken in biotechnology laboratory for further experimental analysis. The plant was ensured to be safe and uninfected. Under running tap water, the leaves were washed to remove dust and other foreign particles and to carefully dry the leaves and keep some new leaves dried under the shadow.

The dried and fresh leaves were crushed into tiny parts, powdered and individually blended in the aforementioned powder samples (30 g) were suspended in ethanol (150 ml) and kept at room temperature for 4 hr. The first blend was then filtered using a Bruckner funnel with what man filter paper No.1 was used and again 75ml ethanol was added at room temperature in the remaining residual powder in the first conical flask and filtered after 24 hours. In a round-bottom flask using a water bath, the first and second blended ethanol solvent evaporated from the filtrate were then space dried at 60ºc up to 20 mg dried weight remaining using water bath. The concentrate was then stored for further research at 4ºC.

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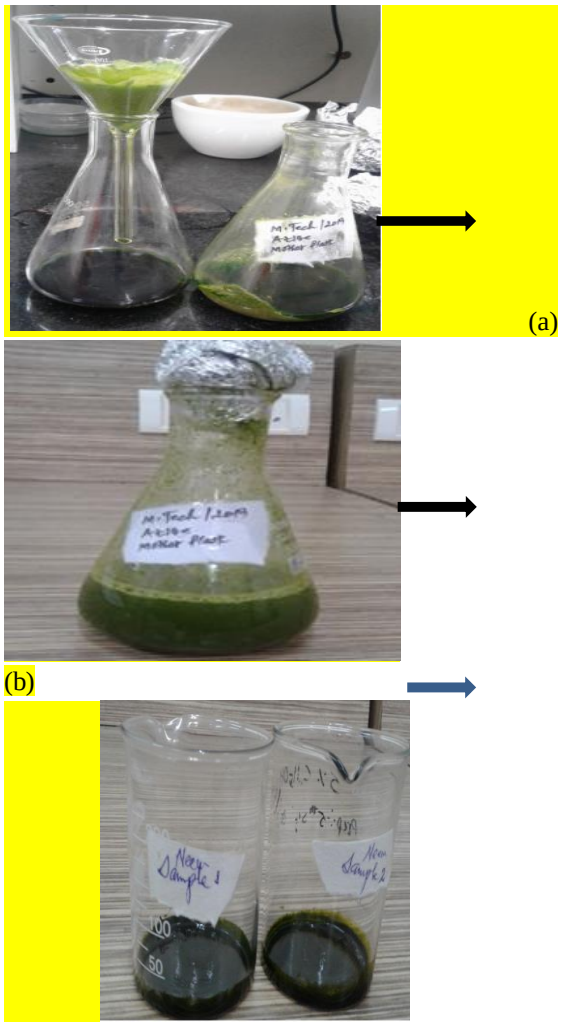

Figure1(a) Ethanol solvent neem leaves powder filtration (b) filtered from solution (c) dried with water bath powder

3.2 Chicken cell line and experimental design:

3.2.1 Molecular gene Characterisation and Biochemical Enzyme Activity

Intestinal Embryo Chicken Cell Line was used in this study. Intestinal embryo chicken's Cell line was purchased from National Center Cell Science NCCS Pune. Experimental procedures were performed in accordance with Cells were allocated into 6 well plates in molecular gene characterization and 6 well plates biochemical enzyme activity. All the treatment groups :-In the Sixth treatment each well plate 400µl cell line, 3ml PRMI Media, and 200 µl Phytic Acid were culture incubated at 37°C and 5% CO2 for 10 days to allow differentiation of chicken cell growth ; culture medium was then added with the corresponding condition medium containing phytic acid in all well plate medium and the first well plate is the control medium no added any supplement, the second well plate added neem extract 50µl ,the third well neem extract 100 µL ,the fourth well plate phytase 50µg ,the fifth well plate phytase 100 µL , and the sixth well plate phytase 50µl plus neem extract 50µl mixed composition . PRMI Media was purchased from Gibco Company once.

3.2.2 Examination of the molecular features and biochemical activity of cell line intestinal embryo chicken

After 10 days of cultivation with phytase and neem leaf extract, the cells centrifuged the culture medium 4000 rpm for 10 minutes and discarded the supernatant, the pellet washed as soon as PBS solutionand4000 rpm centrifuge for 10 minutes. The supernatant was finally withdrawn. The molecular characteristics and biochemical activity of the intestinal chicken cell with 800 µL Trizol reagent and 1ml protein lysis buffer with 100 µL proteas inhibitor were then examined respectively.

3.2.3 Molecular characterisation of gene activities

3.2.3.1 RNA Isolation

The medium-added incubated intestinal chicken cell line leaves 10 days of extract and phytase. Then all samples were homogenized and 4000 rpm centrifuged at 10 minutes. Then the supernatant was discarded with 1ml pippete, the pellet washed with 2ml PBS solution and 4000 rpm centrifuged at10minutes. In 800 µL Trizol reagent, the washing cell pellet dissolved and incubated at room temperature for 10 minutes to enable full dissociation of nucleoprotein complexes. At 15 minutes, after incubation, added 300 µL of chloroform and 7000 rpm of centrifuge. The sample mixture centrifugation should be separated into three phases. Total RNA was contained in the colorless aqueous phase of the TOP layer. Then the aqueous stage was thoroughly carried out using a big pippete in a fresh sterile eppendrof tube. Added 700 µL isopropanol in aqueous stage as inventory solution and blended softly, leave for 10 minutes at room temperature. Next centrifuged the RNA on the side and bottom of the pipe at maximum velocity of 14000 rpm for 15 minutes. Using the pippete, the supernatant was separated in the waste container and the RNA pellet washed at 8000 rpm for 10 min by adding 80 percent ethanol and centrifuge. Air dried RNA pellet for 10 minutes and dissolved next stored in -80 0c refrigerators in 30 µL distilled water.

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3.2.3.2 Estimation of RNA concentration

Estimation of RNA concentration from the RNA isolation following 5 µL of the sample combined with 995 µL of each sample distilled water. Then take the Absorbance reading by 260 nm in the individual samples. The RNA concentration of each sample was calculated by OD reading*40µg / ml*dilution factor of each sample at the end of the reading. Finally, the complete RNA in the incubated chicken cell line is calculated using 4 µL.

3.2.3.3 First Strand cDNA Synthesis

Mix and briefly centrifuge the kit parts after thawing. The first technique added sterile tube reagents to Template RNA calculated from Total RNA 4 g, the second method prepared Master Mix 147 µL/7=21 µL parts of Water 12µl / Each Sample Tube(7), Oligo(dT)18 Primer 1µl / Each Sample Tube,5x Reaction Buffer 4 µL / Each Sample Tube, Ribo Lock Rnase inhibitor (20u/µL) 1 µL / Each sample tube, 10mM dNTP mix 2 µL / Each sample tube, Revert Aid M-MULVRT(200u/µL) 1 µL / Each sample tube and the third process to add 20 µL Master Mix Solution for each sample with master mix control (7 samples). Thermo Fischer Scientific's cDNA kit.

3.2.3.4 Gene Primer Design

A primer is a short synthesized oligonucleotide used in many molecular methods from PCR to DNA sequencing. These primers are designed to have a sequence that is the reverse complement of a DNA region that we want to anneal to the primer sequence analysis model or target. The first you design has an effect on the DNA amplification stage as a whole. DNA polymerases, the enzymes that catalyze DNA replication, can only be initiated by adding nucleotides to the primers. Therefore, an effective amplification of DNA is required A main enzyme called primase, which is a kind of RNA polymerase, must be synthesized before DNA replication can occur, because DNA synthesizing enzymes, called DNA polymerases, can only add new DNA nucleotides to a present nucleotide strand. The gene priming design measures followed by the use of the global web national biotechnology institute center (www.ncbi.nih.gov) to the gene page discovered six cellular regulatory genes in the domestic chicken (Gallus gallus chicken). After that each cellular regulatory gene sequence mRNA was searched, the new sequence was linked and the region of coding clicked. Then pick, copy and place the origin a sequence on the website of Gene Script PCR Primer Design, Paste (sequence space), Pick Primer and Primer Design. After completion of downloading each cellular regulatory gene's forward and reverse sequence, choose the finest primer design.

3.2.3.5 The PCR Amplification and RT-PCR Sequences

The first strand cDNA synthesis product can be used in PCR or qPCR straight. The first strand cDNA synthesis reaction mixture quantity should not exceed 1/10 of the complete amount of PCR reaction. Normally, 2 µL of the first cDNA synthesis response blend is used in 50 µL complete volume as a template for successive PCR.

The PCR cycling parameters were pre-denaturation at 95 ° C for 10 min; 35 cycles (94 ° C for 30 seconds denaturation; 60 ° C for 30 seconds annealing; and 72 ° C extension for 60 seconds) and a final extension at 72 ° C for 5 minutes. The RT-PCR cDNA sequences made by the Delhi Nuclear Medicine and Allied Science Institute (INMAS). Biosystem Applied. CTX Machine CDNA (GenBank mRNA number: Xm 015292141.2 for the p53 gene, Xm 004941288.2 for the p21 gene, Xm 025146627.1 for the ALP gene, Nm 001038693.2 for the G-6-P gene, Nm 204101.1 for the Hexo kinase gene, and Xm 003643958.4 for the p19 gene) has been enhanced by PCR with F1 and R1 primers,F4 and R4 primers, F3 and R3 primers, F4 and R4 primers, F3 and R3 primers, and F4 and R4 primers respectively (Table 4) built on the basis of the 5'- and 3'-Real Time-PCR amplification product sequence data.

All PCR amplification products were electro phoresized to an agarose liquid of 1.2 percent comprising Gel Green Nucleic Acid Gel Stain, 10,000X at the DMSO Institute of Nuclear Medicine and Allied Science (INMAS), Delhi. Applied biological system. CTX Machine and extracted from the agarose gel using a Fast Gene Gel / PCR Extraction Kit (INMAS), Delhi) followed by sequence assessment.

3.2.4 Biochemical enzyme activity

In biochemical enzyme activity, experimental processes were conducted in accordance with the allocation of cells to six wells plate. To assess the phytase and Neem extract to determine the activity of the phosphorylation pathway in the cell line. Phosphorylation is the method of introduction into an organic molecule of a phosphate group. It plays a key role in regulating cellular procedures such as cell apoptosis, cycle, development and signal transduction pathways. Enzymes are proteins in front of protein-based drugs otherwise catalyze drug responses to promote race happy. Enzymes are particularly distinctive, being used highly

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selectively for substrates at home (substance before molecules on which they have an impact) also in the name of the finished harvest they generate. From the moment enzymes are proteins, they are vulnerable before destruction close digestive enzymes in the direction of likely denaturation, regardless of which can promote alteration of their structure. Typically, enzymes have superlative environments (temperatures, pH, etc.) somewhere they run gamely new.

3.2.4.1 Isolation of Protein

The extraction methods followed are described in the samples added in the medium Neem extract and phytase were first incubated on 37ºc in the co₂ incubator for 10 days, mixed with overtaxing, and centrifuged the medium 4000 rpm for 10 min all samples. Then the supernatant was discarded, the pellet washed with 2ml PBS solution and the solution centrifuged again for 10 minutes by 4000 rpm. Protein standards have been prepared with 1ml protein lysis buffer plus 100µl protease inhibitor (cocktail tablet). After centrifuging 10000 rpm for 15 min, it took the supernatant and stored -20ºc fridges to use the estimated concentration of protein and activity of the enzyme.

3.2.4.2 Procedures Protein Estimation by Lawry Method

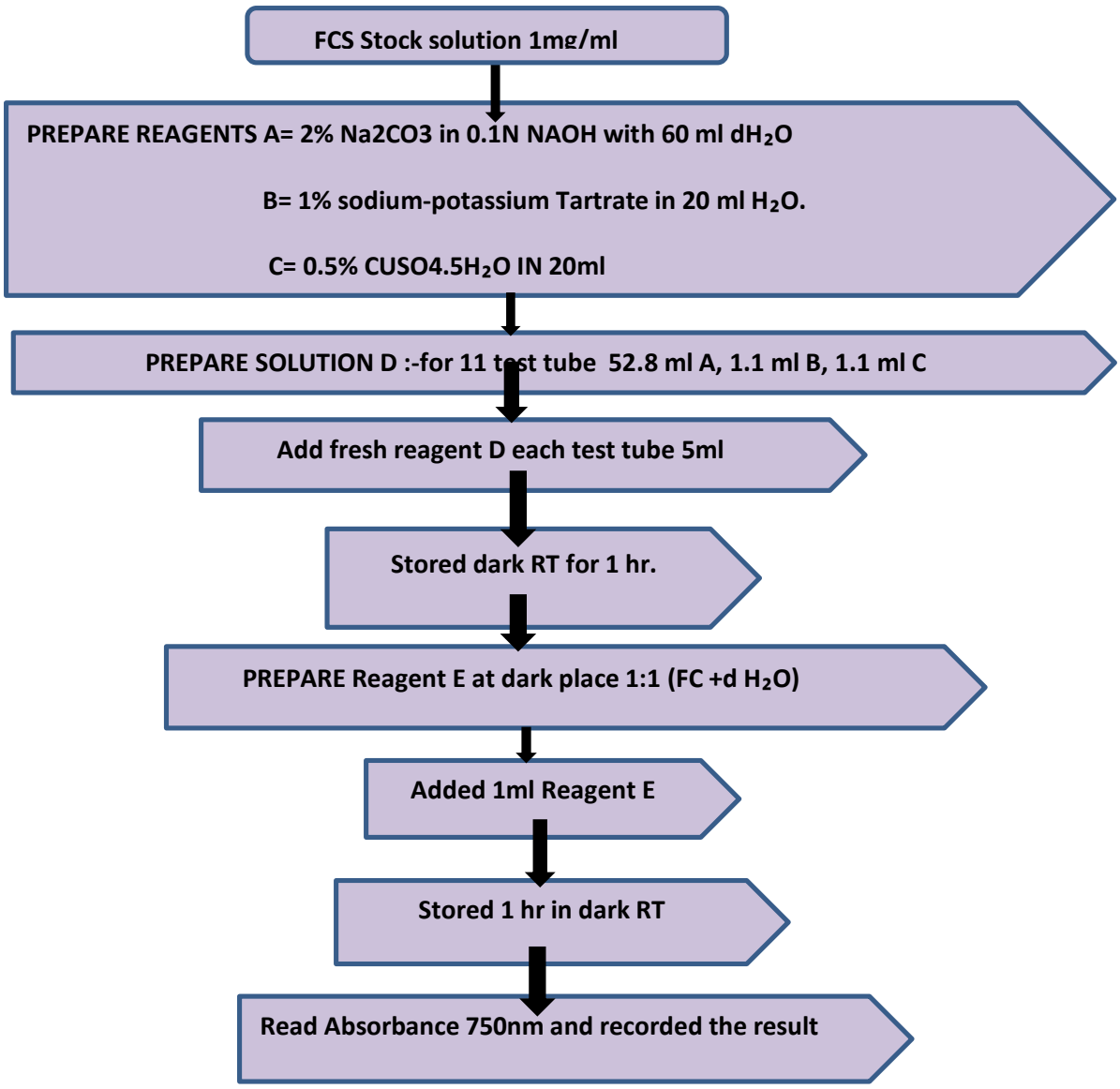

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Figure 2The procedure of the estimation protein concentration from the isolated protein by Lowry method

3.2.4.3 Alkaline phosphatase KIT (ALP)

A minor protein on the brush's boundary, ALP is a very vibrant enzyme that can easily be measured in tissue and cell homogeneity, making it the preferred marker for early research in-vitro intestinal cell growth (Moog, Fetal., 1953, Koldovsky.0etal.,1969, HenningS.Jetal.,1985).ALP activity has been used to mean frequently mobile intestinal epithelial strains, but the intestinal ALP can be handled with isoforms found in other tissues,

cells and body fluids. cDNA and genes coding for a distinctive ALP bureaucracy have been cloned and can be used to collect particular samples to that extent theoretically. Enzymes were the largest monoclonal antibodies in the intestinal system and each pig was distinctive (Quaroni, A et al., 1985).

The alkaline phosphatase kit used to evaluate enzyme activity was performed at 100µg per protein blended sample With 900µl of alkaline phosphatase buffer kit and 50µl of Para Nitro phenol (substrate) added 5µl of ALP enzyme next to incubated overnight at 370c, yellow-colored enzyme activity was shown to be present and absorbance read at 405 nm.

III. STATISTICAL ANALYSIS

The cellular regulatory gene activities and enzymatic activity concentration of the intestinal Embryonic broiler chicken cell line were analyzed quantitatively by two-way ANOVA test and excel respectively.

IV. RESULTS

To explain the RT-PCR Results. The cDNA sequences of the RT-PCR done- Institute of Nuclear Medicine and Allied Science (INMAS), Delhi. Applied Biosystem. CTX Machine a highly sensitive technique for the detection and quantitation of mRNA (messenger RNA). The technique consists of two parts: The synthesis of cDNA (complementary DNA) from RNA by reverse transcription (RT), and the amplification of a specific cDNA by the polymerase chain reaction (PCR) in chicken cell line the different regulatory gene to correlated the average CT Value target untreated and in the home keeping gene (GADPH) target untreated with the average CT Value target treated and in the home keeping gene (GADPH) target treated compared with the fold change functional gene expression product with in each samples treatment (control, Neem extract and phytase supplement) and within six cell regulatory gene product in chicken cell line cultured with phytase and neem leaves extract.

5.1 The role and functions of regulatory gene protein

A gene that regulates, or regulates is a gene that regulates the expression of one or more other genes. Regulatory sequences encoding regulatory genes are often 5' to the point of transcription of the gene they control. Regulatory sequence checks when and where protein expression happens. The promoter and enhancer regions regulate the gene transcription into a modified pre mRNA to remove introns and add a 5 ' cap and a dark gray poly-A tail.

Gene expression regulation or gene regulation encompasses a broad variety of mechanisms that cells use to boost or reduce the output of particular gene products (protein or RNA). The protein function depends on the form of the protein. The amino acid order determines the shape of the protein. The protein information is encoded in the cell's DNA. When a protein is generated and this copy is transferred to a ribosome, a copy of the DNA is made.

5.1.1 P53 gene activity

The critical event that leads to the activation of p53 is its N-terminal domain phosphorylation. The N-terminal transcriptional activation domain includes a big amount of phosphorylation locations and can be regarded as the primary target for kinases of protein that transmit stress signals.

P53 is activated in reaction to a multitude of stressors, including but not restricted to damage to DNA, oxidative stress,(Hendriks, G et al., 2015) . Activated p53 binds DNA and activates multiple gene expression, including microRNA miR34a, National Biotechnology Information Center (Su, W et al., 2014). The RT-PCR result of p53 gene in the Average CT(target, untreated)value and Average CT (ref-GAPDH, untreated) with the average target treated cycling threshold and Average CT(ref-GAPDH, treated) values were almost the same CT value number in control treatment and (in neem 50 µL, neem 100 µL) decreased , phytase 50 µL, phytase 100 µL ,and phytase 50 µL plus neem 50 µL sample treatment cycling threshold value increased and to the reverse the fold change functional gene expression product(in neem 50 µL, neem 100 µL) increased ,other sample treatment decreased respectively.

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5.1.2 The P21 Gene activity

The p21 gene includes several p53 response elements that mediate direct binding of the p53 protein, leading in the p21 protein encoding gene being transcriptionally activated. P21 (CDKN1A) belongs to a cell cycle-dependent family of kinase inhibitors. P21 modulates distinct mechanisms, including cell development, differentiation (CU, P., & GAO, C. F. (2009).) and apoptosis

P21 is a persuasive kinase inhibitor (CKI) dependent on cycline. The protein p21 (CIP1/WAF1) also inhibits the leisure value of cyclin-CDK2, -CDK1, moreover -CDK4/6 complexes, while a group series director next to G1 also works in this manner. Cdks gesture the chamber through phosphorylation with the aim of organizing it just before the lob interested in the chamber cycle after that point. Cycling-dependent protein kinases, as their call indicates, are dependent next to cyclins, an extra lecture on authoritarian proteins. Cyclins join Cdks, activating additional molecules near phosphorylate. P21 is expressed in a constitutive and cell cycle-dependent way in the proliferation of cells at a basal level, (Siddik, Z. H. (Ed.). (2010). Therefore, whenthe RT-PCR result of p21 gene in the Average CT(target, untreated)value and Average CT (ref-GAPDH, untreated) with the average target treated cycling threshold and Average CT(ref-GAPDH, treated) values were almost the same CT value number in control treatment and (in neem 50 µL, neem 100 µL , phytase 50 µL, phytase 100 µL ware increased, and phytase 50 µL plus neem 50 µL sample treatment cycling threshold value was decreased and to the reverse the fold change functional gene expression product in neem 50 µL, neem 100 µL , phytase 50 µL, phytase 100 µL decreased, sample treatment phytase 50 µL plus neem 50 µL was increased respectively.

5.1.3 The glucose-6- phosphatase

This enzyme, which is vigorously trendy for all cell kinds and purposes, is elaborate when carbohydrates are dissolved as normal. This enzyme helps prevent premature destruction of crimson blood cells.G6PD deficiency is a genetic idiosyncrasy with an inadequate amount of glucose6phosphate dehydrogenase (G6PD) common in the blood to produce a fashionable result. This is an enormously precious enzyme (or protein) with the purpose of regulating numerous biochemical responses within the body. It is accepted that this primitive destruction of ruby blood cells has the status of hemolysis, next it can lead to hemolytic anemia in the end guide. The underlying trigger has long been handled before it has been resolved, symptoms of G6PD deficiency usually pass away surrounded by a few weeks. G6PD deficiency is a genetic clause for which parents close their kid are recognized as single otherwise mutually.

The RT-PCR result of the glucose-6- phosphatase gene activity in the Average CT(target, untreated) value and Average CT (ref-GAPDH, untreated) with the average target treated cycling threshold and Average CT(ref-GAPDH, treated) values were almost the same CT value number in control treatment and in neem 50 µL, neem 100 µL were increased, phytase 50 µL, phytase 100 µL, phytase 50 µL plus neem 50 µL sample treatment cycling threshold value was decreased and to the reverse the fold change functional gene expression product in neem 50 µL, neem 100 µL decreased and phytase 50 µL, phytase 100 µL, phytase 50 µL plus neem 50 µL were highly increased respectively.

5.1.4 The Hexo Kinase Gene activity

A hexokinase is an enzyme that facilitates the formation of hexose phosphorylates. Hip the majority of humans, glucose, in relation to glucose6phosphate, is the most significantly important product. During the firstly pace of glycolysis, the glucose mob is phosphorylated. Phosphorylation is the handle of tallying a phosphate assemble near a molecule consequential beginning ATP. The upshot occurs amid the relief of the enzyme hexokinase, an enzyme so as to catalyze the phosphorylation of a lot of six-membered glucose-likechimestructures. Hexokinase. The chief movement in vogue glycolysis is a priming reaction; someplace a phosphate congregate is extra on the road to glucose via ATP. This outcome is notable instead of its capability on the way to con glucose contained by the cell. Hexokinase activates glycolysis near phosphorylating glucose. The RT-PCR result of the glucose-6- phosphatase gene activity in the Average CT(target, untreated) value and Average CT (ref-GAPDH, untreated) with the average target treated cycling threshold and Average CT(ref-GAPDH, treated) values were almost the same CT value number in control treatment and in neem 100 µL phytase 50 µL, were increased, neem 50 µL, , phytase 100 µL, phytase 50 µL plus neem 50 µL sample treatment cycling threshold value was decreased and to the reverse the fold change functional gene expression product in neem 100 µL phytase 50 µL, decreased and neem 50 µL, phytase 100 µL, phytase 50 µL plus neem 50 µL were highly increased respectively.

5.1.5 The P19 Gene activity

The p19 protein binds to double-stranded RNAs that function as short interfering RNA (siRNA) and

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is specialized for the 21-nucleotide product of the enzyme DCL4 (a member of a family of plant enzymes with homology to Dicer) (Csorba, T et al., 2009). Consequently, the selective sparing of its repressor from the overall sequestration of miRNA by p19 reduces cellular AGO1 concentrations and constitutes an extra mechanism by which p19 inhibits silencing (Hipper, C et al., 2013), Várallyay E et al., 2010). The two mechanisms are mutually autonomous and can be abrogated selectively (Alers, S et al., 2012).

The RT-PCR result of the P19 Gene activity in the Average CT(target, untreated) value and Average CT (ref-GAPDH, untreated) with the average target treated cycling threshold and Average CT(ref-GAPDH, treated) values were the same CT value number in control sample treatment and in neem 50 µL, neem 100 µL, phytase 50 µL, phytase 100 µL, phytase 50 µL plus neem 50 µL sample treatment cycling threshold value were decreased and to the reverse in neem 50 µL, neem 100 µL, phytase 50 µL, phytase 100 µL, phytase 50 µL plus neem 50 µL the fold change functional gene expression product were increased respectively.

5.1.6 The Alkaline phosphatase Gene activity

Alkaline phosphatase is an ubiquitous membrane-bound glycoprotein that catalyzes the hydrolysis of phosphate monoesters at basic pH values. Alkaline phosphatases are plasma membrane-bound glycoproteins. These enzymes are widely distributed in nature, including prokaryotes and higher eukaryotes with the exception of some higher plants. Alkaline phosphatase forms a large family of dimeric enzymes, usually confined to the cell surface hydrolyzes various monophosphate esters at a high pH optimum with release of inorganic phosphate.

The gene encoding for intestinal ALP (IAP) is a member of the gene family mapping to the long arm of chromosome 2, IALP is partially heat-stable isozyme present at high levels in intestinal tissue. In contrast to the other ALP isoenzymes, the carbohydrate side-chains of IAP are not terminated by sialic acid.

The RT-PCR result of the Alkaline phosphotasegene activity in the Average CT(target, untreated) value and Average CT (ref-GAPDH, untreated) with the average target treated cycling threshold and Average CT(ref-GAPDH, treated) values were the same CT value number in control sample treatment and in neem 50 µL, neem 100 µL, phytase 50 µL, phytase 100 µL, phytase 50 µL plus neem 50 µL sample treatment cycling threshold value were decreased and to the reverse the fold change functional gene expression product in neem 50 µL, neem 100 µL, phytase 50 µL, phytase 100 µL, phytase 50 µL plus neem 50 µL were highly increased respectively.

Table:-1 The RT-PCR results of average cycling threshold value of the target, untreated samples with the average cycling threshold ref-GAPDH, untreated (home keeping gene) compared with the fold change gene expression effect. The cDNA sequences of the RT-PCR done- Applied Biosystem. CTX Machine.

P53	Average CT(target, untreated)	Average CT(ref-GAPDH, untreated)	Average CT(target, treated)	Average CT(ref-GAPDH, treated)	DeltaDeltaCT	Fold change Gene Expression
A Untreated Control	27.20	23.14	27.20	23.14	0	1
B Neem -50			27.16	23.14	0.05	1.036587801
C Neem -100			27.13	23.08	0.02	1.015021102
D Phytase-50			27.56	23.19	-0.31	0.805707793
E Phytase-100			27.52	23.10	-0.35	0.784383301
F Neem(50) + Phytase (50)			27.77	23.20	-0.50	0.707847127
P21	Average CT (target, untreated)	Average CT (ref-GAPDH, untreated)	Average CT(target, treated)	Average CT(ref-GAPDH, treated)	DeltaDeltaCT	fold change Gene Expression
A Untreated Control	27.10	23.14	27.10	23.14	0.007431391	1.005164337
B Neem -50			27.16	23.21	0.02	1.015255133
C Neem -100			27.13	23.11	-0.05	0.966947671
D Phytase-50			27.16	23.24	0.04	1.026923431
E Phytase-100			27.44	23.17	-0.30	0.812044519

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F Neem(50) + Phytase (50)			26.97	23.27	0.27	1.204838244
Glucose-6-Phosphatase	Average CT (target, untreated)	Average CT (ref-GAPDH, untreated)	Average CT(target, treated)	Average CT(ref-GAPDH, treated)	DeltaDeltaCT	Fold change Gene Expression
A Untreated Control	27.32	23.14	27.10	23.14	0.225820305	1.169441995
B Neem -50			27.16	23.21	0.24	1.181181966
C Neem -100			27.25	23.11	0.04	1.030462675
D Phytase-50			25.16	23.24	2.26	4.779029025
E Phytase-100			25.25	23.17	2.11	4.310991272
F Neem(50) + Phytase (50)			24.52	23.27	2.94	7.659384077

Alkaline Phosphatase	Average CT (target, untreated)	Average CT (ref-GAPDH, untreated)	Average CT(target, treated)	Average CT(ref-GAPDH, treated)	DeltaDeltaCT	fold change Gene Expression
A Untreated Control	27.87	23.14	27.87	23.14	0.005820305	1.004042477
B Neem -50			27.80	23.21	0.15	1.109747328
C Neem -100			27.79	23.11	0.05	1.03763011
D Phytase-50			25.16	23.21	2.78	6.852913751
E Phytase-100			25.21	23.19	2.72	6.579690129
F Neem(50) + Phytase (50)			24.53	23.20	3.41	10.60908541
Hexo kinase	Average CT(target, untreated)	Average CT(ref-GAPDH, untreated)	Average CT(target, treated)	Average CT(ref-GAPDH, treated)	DeltaDeltaCT	fold change Gene Expression
A Untreated Control	28.85	23.14	28.85	23.14	0.005820305	1.004042477
B Neem -50			28.83	23.21	0.10	1.07194526
C Neem -100			28.92	23.11	-0.09	0.937719529
D Phytase-50			26.87	23.24	2.08	4.21846324
E Phytase-100			26.25	23.17	2.64	6.224766159
F Neem(50) + Phytase (50)			26.23	23.27	2.76	6.760961275
P19	Average CT(target, untreated)	Average CT(ref-GAPDH, untreated)	Average CT(target, treated)	Average CT(ref-GAPDH, treated)	DeltaDeltaCT	fold change Gene Expression
A Untreated Control	27.43	23.14	27.43	23.14	0.007515044	1.005222622
B Neem -50			27.24	23.21	0.27	1.207418636
C Neem -100			27.17	23.11	0.24	1.182297721
D Phytase-50			27.37	23.24	0.16	1.117803928
E Phytase-100			27.23	23.17	0.24	1.180758876
F Neem(50) + Phytase (50)			26.90	23.27	0.67	1.589885779

Table :-2 The two-way ANOVA to express to the sum and mean value within the samples(control, Neem 50µl, Neem 100µl ,Phytase 50µl, Phytase 100µl , Phytase 50µl+ Neem 50µl) and on the other hand with cellular regulatory genes(p53,P21,G-6-P, ALP ,H.K.andP19).

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		Two-way Anova				
		Sample				
Treatment	control	Neem 50	Neem 100	Phytase 50	Phytase 100	Phytase 50+Neem 50
p53	1	1.036587801	1.015021102	0.805707793	0.784383301	0.707847127
p21	1.005164337	1.015255133	0.966947671	1.026923431	0.812044519	1.204838244
ALP	1.004042477	1.109747328	1.03763011	6.852913751	6.579690129	10.60908541
G-6-P	1.169441995	1.181181966	1.030462675	4.779029025	4.310991272	7.659384077
Hexokinase	1.004042477	1.07194526	0.937719529	4.21846324	6.224766159	6.760961275
P19	1.005222622	1.207418636	1.182297721	1.117803928	1.180758876	1.589885779
sum	6.187913908	6.622136124	6.170078808	18.80084117	19.89263426	28.53200191
AVERAGE	1.031318985	1.103689354	1.028346468	3.133473528	3.315439043	4.755333652

Table: -3 the summary of the six samples indicates the sum, average mean, and variance within two-factor Without Replication.

Anova: Two-Factor Without Replication				
SUMMARY	*Count*	*Sum*	*Average*	*Variance*
1	5	4.349547124	0.869909425	0.021634959
1.005164337	5	5.026008998	1.0052018	0.019800181
1.004042477	5	26.18906673	5.237813346	16.98555362
1.169441995	5	18.96104902	3.792209803	7.660674205
1.004042477	5	19.21385546	3.842771093	7.611919852
1.005222622	5	6.27816494	1.255632988	0.036007649
Neem 50	6	6.622136124	1.103689354	0.006026497
Neem 100	6	6.170078808	1.028346468	0.007190454
Phytase 50	6	18.80084117	3.133473528	6.327652341
Phytase 100	6	19.89263426	3.315439043	7.468146078
Phytase 50+Neem 50	6	28.53200191	4.755333652	17.14613715

Table:-4 The Statistical analysis describe the highly significance in source of variation in the ROWs(The cellular regulatory gene) AND COLUMNS(Samples) and error Founded the sum square (SS),Degree of freedom (df),Mean of square (MS), F-Value (F), and the P-value ($p<0.05$) highly significance

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ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Rows	86.20340132	5	17.24068026	5.028463348	0.003820411	2.710889837
Columns	60.77000059	4	15.19250015	4.431085606	0.009996198	2.866081402
Error	68.57236126	20	3.428618063			
Total	215.5457632	29				

5.2 Alkaline phosphatase KIT (ALP)

ALP activity has been used to mean frequently mobile intestinal epithelial strains, but the intestinal AP can be handled with isoforms found in other tissues, cells and body fluids. cDNA and genes coding for a distinctive ALP bureaucracy have been cloned and can be used to collect particular samples to that extent theoretically. The alkaline phosphatase kit used to evaluate enzyme activity was performed at 100µg per protein blended sample With 900µl of alkaline phosphatase buffer kit and 50µl of Para Nitro phenol (substrate) added 5µl of ALP enzyme next to incubated overnight at 37ºc, yellow-colored enzyme activity was shown to be present and absorbance read at 405 nm.

Table:-5 alkaline Phosphatase Enzyme Activity Reading by 570nm in the positive control 5nm paranitrophenol.

	OD Reading 570nm
Blank	0
Positive Std control 0.150 ALP activity	1.5
Neem 50 ALP activity	0.035
Neem 100 ALP activity	0.038
Phytase 50 ALP activity	1.7
Phytase 100 ALP activity	2.4
phytase 50 +Neem 50 ALP activity	2.83
Sum	8.503
Average	1.214714286

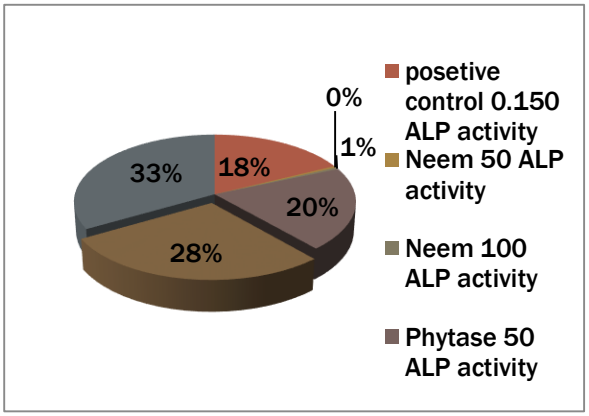

Fig:-4 the pie chart showed the variation of the positive control with each treatment groups of alkaline Phosphatase enzymatic activity in percent.

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V. DISCUSSION

Phytate (6-myo-inositol phosphate) is the leading foundation of P current in plant kernels and is habitually an out of sorts' obtainable foundation of P for monogastric. In totaling, phytate can interrelate by additional nutrients present-day in the nourishment, thus decreasing inorganic and protein immersion and growing endogenous damages. In line for to these destructive properties, phytate is accepted as an important antinutrient in broiler nourishments (Cowieson A. J et al., 2008). The objective of this research was to explore changes in the chicken embryonic cell line in the genome profiling with gene expression under circumstances of the metabolic pathway of phosphorus as seen in the activity of molecular genes and biochemical enzyme. During, in Chicken Cell line P53 gene expression product was statistically not significantly induced in response to Neem alone treatment, whereas in Phytase treatment, there was dose dependent decrease by 0.2 fold with and without Neem. In Chicken Cell line P21 gene expression was statistically not significantly induced in response to Neem alone treatment, whereas in Phytase treatment, there high dose showed decrease by 0.2 fold percent without Neem and increase by 0.2 fold percent with Neem• In Chicken Cell line Glucose 6 phosphatase gene expression was statistically not significantly induced in response to Neem alone treatment, whereas in Phytase treatment, there was Dose dependent increase by 4.3 to 4.7 fold without Neem whereas 7.6 fold induction was found with Neem. In Chicken Cell line Hexokinase gene expression was statistically not significantly induced in response to Neem alone treatment, whereas in Phytase treatment, there was dose dependent increase by 4.2 to 6.2 fold without Neem whereas 6.7 fold induction was found with Neem. In Chicken Cell line P19 gene expression was statistically significantly induced in response to Neem alone treatment, Phytase treatment, there was dose dependent increase with and without Neem. In Chicken Cell line Alkaline Phosphatase gene expression was not significantly induced in response to Neem alone treatment, whereas in Phytase treatment, there was dose dependent increase by 6.5 to 6.8 fold without Neem whereas 10.6 fold induction was found with Neem. In the p53 and p21 gene cycling threshold value the cDNA amplification increases in treatment group control, neem 50µl, neem 100 µL, phytase 50 µL, phytase 100 µL and neem 50 µL plus phytase 50 µL; to the reverse the functional protein gene expression decreased respectively and the ALP, G-6-P, Hexo kinase and p19 gene the cycling threshold value the cDNA amplification decreased in treatment group control, neem 50 µL, neem 100 µL, phytase 50 µL, phytase 100 µL and neem 50 µL plus phytase 50 µL , and the reciprocal the functional protein gene expression increased, Then the cDNA amplification cycling took short time was the functional protein gene expression higher product. The results was statistically high significant difference the control and all treatment group p-value ($p<0.005$). And all of the cellular regulatory gene p53, p21, p19, ALP,G-6P,Hexa Kinase) also was statistically high significant difference($p<0.005$).

In chicken cell line the enzyme activity Alkaline phosphatase shows the neem samples are less performance to compare positive control, the phytase 50 sample equal to positive control and the other phytase 100 µL and neem mixed with phytase 50 µL performance high than to positive control.

Then, Biochemical Estimation of Alkaline phosphatase shows that Phytase and Neem induces Alkaline Phosphatase due to generation of more Inorganic phosphate by Enzyme. Neem also plays an important position in strengthening the immune gadget of the frame. Enzymes were the largest monoclonal antibodies in the intestinal system and each pig was distinctive(Quaroni, A. (1986) Growth in antibodies in opposition to new castle and infectious bursal sickness viruses had been found when neem is incorporated in poultry feeds (Durrani Z (2008).Water based totally extract (10%) of neem leaves is reported to have anti-viral houses in opposition to, chook pox, infectious bursal sicknesses (IBD) and Newcastle sickness virus (NDV) and it notably enhances the antibodies manufacturing in opposition to the IBD and NDV (Sadekar RD et al., 1998). The prevailing have a look at turned into consequently designed to file the impact of A. indica on immunity of industrial broilers against New castle and infectious bursal sickness.

Neem, Supplementation, increase, Broiler, physiology, medicine, Antibacterial, historic. introduction many man made capsules and growth promoters are supplemented to the broilers to impact fast boom, however their use have proven many risks like excessive fee, destructive facet effect on fitness of birds and lengthy residual residences and many others. So, scientists are once more targeting the usage of our historic medicinal device to discover beneficial herbs and flora, which can be accurately used to boom the manufacturing. Certainly one of such vegetation, Neem (Azadirachta indica) is an indigenous plant of Asian subcontinent acknowledged for its useful medicinal residences considering that ancient instances. Neem has attracted world huge

prominence due to its giant variety of medicinal homes like antibacterial, antiviral, anti-fungal, antiprotozoal, hepatoprotective and numerous different properties without displaying any detrimental impacts (Kale, B.P et al., 2003).Also, neem promotes boom and feed efficiency of birds due to its antibacterial and hepato protective properties

VI. CONCLUSION

The above conclusion indicate that in intestinal Broiler Chicken cells Phytase and Neem involvement of Cell Cycle regulatory genes P53 and p21 are involved to lesser extent in Phosphate Metabolism, whereas Glucose 6 phosphate, Hexokinase and Alkaline Phosphatase are the direct targets of Phosphate Metabolism.

Hence use of Phytase and Neem extract in diet of Chickens can be beneficial to generate less Phosphorous waste and protecting

Pollution and improve the utilization, digestion of feed ingredients, reduced wastage of other feeds nutrient and reduced costs of supplementary feed in good quality of chicken growth. Now that the gene expression and enzymatic activity with the sample of phytase and neem extract done in vitro chicken cell line successfully achieved, in the future recommend will be practice in vivo methods for the demonstration of all regulatory genes it should be possible to obtain further insights in the function of phosphorylation pathway in the metabolism of cells.

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