MOLECULAR ASSESSMENT OF THE IMMUNOMODULATORY EFFECTS OF COMMON PHYTOGENIC EXTRACTS ON BROILER CHICKENS ASSESSED BY EXPRESSION PROFILES OF CERTAIN PRO- AND ANTI-INFLAMMATORY GENES

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Executive Summary

The Nigerian poultry industry, a cornerstone of national food security, is critically hampered by high production costs, disease outbreaks, and the escalating threat of antimicrobial resistance (AMR) linked to the prophylactic use of antibiotics. There is a growing need for alternative strategies to replace synthetic components (antibiotics) with natural antimicrobial compounds in poultry diets to improve growth performance, enhance immunity, reduce oxidative stress, and improve gut histology and digestibility. The extensive use of synthetic antimicrobials in poultry diets promotes the growth of drug-resistant microbes, affecting both human and bird health. The major challenges in the broiler industry are disease pressure, antibiotic abuse, and rising input costs. This project will address these critical challenges in broiler chicken production in Nigeria by scientifically evaluating readily available indigenous plants (phytogenics) as natural immunomodulators and growth promoters. The study will assess the impact of phytogenics; Drumstick tree (*Moringa oleifera)*, Neem (*Azadirachta indica), Garlic* (*Allium sativum*), scent leaf (*Ocimum gratissimum*), bitter leaf (*Vernonia amygdalina*), lemon grass (*Cymbopogon citratus*), clove (*Syzygium aromaticum*), eucalyptus (*Eucalyptus globulus*), cinnamon (*Cinnamomum verum*), Tumeric (*Curcuma longa*), and ginger (*Zingiber officinale)* on the growth, carcass characteristics, and immune status of broiler chickens. Advanced molecular biotechnologies (gene expression profiling and quantitative real-time PCR) will elucidate the mechanism of action of pro-inflammatory (IL-1β, IL-6, TNF-α, IFN-γ) and anti-inflammatory (IL-4, IL-10, TGF-β) cytokine genes in the spleen and cecal tonsils that are involved in disease susceptibility and immune status of the chickens. The core of this research will move beyond conventional growth-performance metrics and provide robust, molecular-level evidence of their effects, thereby providing scientific validation for a sustainable, homegrown solution to reduce antibiotic dependency. This will also develop evidence-based

recommendations for the Nigerian poultry sector, ultimately enhancing productivity, profitability, and public health. This innovative approach promises significant economic and scientific impact by bridging traditional knowledge with advanced molecular biology. Thus, this study will provide a cost-effective and scientifically tested phytogenic extract mixture (organic growth promoter and immune booster (Nasboost®)) that will sustainably enhance poultry health and productivity (that is, reduce morbidity and mortality, and improve growth), thereby reducing reliance on antibiotics and importation of poultry products. This will further enhance household protein and food security and reduce poverty in Nigeria.

Introduction

Background of the study

The global poultry industry faces mounting challenges driven by the quest for enhanced productivity. The sector is very crucial in Nigeria, contributing significantly to the economy, food security, and livelihoods of millions. However, productivity remains challenged by disease outbreaks, antibiotic resistance, and inefficient feed conversion, compromised immune resilience, and increasing consumer demand for antibiotic-free poultry products (Olowoyeye, 2025). In addition, stressors associated with high-density rearing, vaccination, transportation, and environmental extremes can disrupt immune homeostasis, leading to increased production losses due to infection and chronic inflammation. The economic and nutritional importance of broiler production in Nigeria cannot be overemphasized, due to the rapidly urbanizing population and burgeoning demand for affordable protein. Traditionally, subtherapeutic antibiotic use in poultry diets was considered essential for maximizing growth and controlling disease; however, widespread antibiotic administration (projected to reach over 105,000 metric tons in food-producing animals by 2030) has driven a critical surge in multidrug-resistant (MDR) pathogens (antimicrobial resistance (AMR)) with direct implications for both animal and human health (Mulchandani *et al*., 2023). Woc-Colburn and Bobak (2009) reported that the multidrug-resistant strains, *Salmonella typhimurium* and *Campylobacter jejuni* identified in livestock, are the primary cause of infection in humans. Akosile *et al*. (2023) also reported that antibiotic cross-resistance can develop in humans because antibiotics used in humans and animals are chemically similar.

Regulatory authorities such as the European Union have banned the use of antibiotic growth promoters (AGPs) in poultry production, and similar restrictions are spreading globally (European Union, 2005). This regulatory shift propelled the poultry industry towards safer, sustainable alternatives with equivalent or superior immunological and production outcomes. This has led to an intensified search for natural alternatives (phytogenics) that can simultaneously support growth and robust immune responses in chickens.

Phytogenic feed additives—encompassing herbs, spices, and their aqueous or essential oil extracts—have rapidly emerged as promising alternatives to antibiotics. These botanical extracts are rich in bioactive compounds, including polyphenols, flavonoids, terpenes, saponins, and alkaloids, which are credited with immunomodulatory, antimicrobial, anti-inflammatory, and antioxidant properties (Aminullah *et al*., 2025). Among these, compounds like carvacrol (oregano), thymol (thyme), cinnamaldehyde (cinnamon), curcumin (turmeric), eugenol (clove), and cineole (sage, rosemary) have garnered attention for their direct regulatory effects on immune pathways, including modulation of pro- and anti-inflammatory cytokine gene expression in poultry (Huang and Lee, 2018).

Recent scientific reviews and experimental studies consistently report that supplementation with phytogenic aqueous extracts such as sage (*Salvia officinalis),* oregano *(Origanum vulgare),* thyme *(Thymus vulgaris),* cinnamon *(Cinnamomum verum),* and ginger *(Zingiber officinale)* enhances immunological endpoints in broilers. Ceylan *et al*. (2025) observed increased antibody titers, improved ratios of immunoglobulin classes (IgA, IgG, IgM), reduced serum cholesterol, moderated cytokine gene expression, and shifts in gut microbiota favouring beneficial over pathogenic species, with some extracts outperforming antibiotics under certain challenge models.

At the molecular level, dietary phytogenics can modulate expression of proinflammatory and anti-inflammatory cytokines both in local gut-associated lymphoid tissues and systemic immune organs (Huang and Lee 2018). This modulation is crucial as chronic activation of proinflammatory mediators can lead to intestinal barrier dysfunction, immunosuppression, and metabolic burden, jeopardizing growth and feed conversion. Conversely, balanced expression profiles promote enhanced disease resistance, productive efficiency, and tolerance to environmental stressors (Aminullah *et al*., 2025).

Immunomodulation is described as a change in the indicators of cellular and humoral immunity and nonspecific defence factors (Bakuridze *et al*., 1993). It can present as immunosuppression (substances that inhibit the immune system) or immunostimulation (substances that activate or induce the mediators or components of the immune system), thus regulating or altering the scope, type, duration, or competency of the immune response (Jantan *et al*. 2015). Immune response (*Ir*) genes are defined as antigen-specific genes that control the ability of an animal to raise an immune response, either humoral or cellular to a particular antigen (Berzofsky, 1980). This includes Major Histocompatibility Complex (*MHC I, II*, and III), Interleukins (IL–6, IL-β), Tumor Necrosis Factor (TNF-α), cluster of differentiation (CD-14), and Toll-like Receptor (*TLR-4*), which are responsible for conferring innate immunity. They code for a set of cytokine or anti-inflammatory response complement proteins (C1-C4) that adhere to pathogens and cytokines (interferons and chemokines) that attract immune cells to the site of infection. The relative expression of interleukins (IL)-1β, IL-2,

IL-6, and IL-17 genes was explored in response to coccidial challenge in Kadaknath, Cari-Vishal and Cobb broiler chickens using quantitative PCR (Thakur *et al.* 2020), and it was concluded that the differential expression of cytokine genes in the three genetic groups showed different degree of mucosal immune response to *Eimeria* infection. Research increasingly demonstrates that blends of phytogenic compounds have superior efficacy compared to single-compound supplementation, owing to additive or synergistic actions on multiple physiological targets (Ceylan *et al*., 2025). This synergy is likely due to diverse bioactives acting across different cellular and molecular targets: disrupting pathogens, enhancing barrier integrity, stimulating digestive secretion, and modulating immune responses. However, antagonism can also occur, making optimization and validation essential.

Statement of the problem

The use of antimicrobials in poultry production contributes to antimicrobial resistance and poses significant risks to animal and human health. Phytogenics—plant-derived bioactive compounds—have gained attention as natural immunomodulators in poultry production. They have also been reported to have antimicrobial, anti-inflammatory, and antioxidant properties. Despite these promising outcomes, knowledge gaps persist. While most studies focus on their use as feed additives, the potential of aqueous extracts administered via drinking water remains underexplored. This route offers practical advantages, especially in Nigeria, where feed formulation may be inconsistent. Understanding the molecular basis of their effects can pave the way for antibiotic-free poultry farming. Scientific validation of these phytogenics, especially in their aqueous forms (which facilitate field application and local processing), is crucial for their commercial adoption and optimization in broiler production systems. Furthermore, there is inadequate comparative data on the specific immunomodulatory properties of commonly used aqueous phytogenic extracts in broilers, especially regarding dose-response, synergistic versus antagonistic effects, and their impact on the expression of both pro- and anti-inflammatory cytokine genes. Another critical knowledge gap is the precise mechanisms by which these extracts modulate the immune system, specifically in terms of their impact on the gene expression profiles of key proinflammatory and anti-inflammatory cytokines in broilers. Furthermore, the importance of dose determination and blend optimization cannot be overemphasized as excessively high doses or certain combinations may reduce immunocompetence.

Therefore, this comprehensive and standardized study using molecular technology will establish precise expression profiles for key immune genes in broilers fed with well-defined phytogenic aqueous extracts. It will offer a vital and rational pathway to replacing Antibiotic Growth Promoters in broiler diets in Nigeria. In other words, this research will directly address the urgent need for

science-based, naturally derived, and industry-scalable interventions in modern poultry production and translate botanical promise into practical and repeatable results in Nigeria and comparable global poultry systems.

Objectives of the Research

The major objective of this research will be to determine and compare the immunomodulatory effects of aqueous extracts from Drumstick tree (*Moringa oleifera)*, Neem (*Azadirachta indica)*, *Garlic* (*Allium sativum*), scent leaf (*Ocimum gratissimum*), bitter leaf (*Vernonia amygdalina*), lemon grass (*Cymbopogon citratus*), clove (*Syzygium aromaticum*), eucalyptus (*Eucalyptus globulus*), cinnamon (*Cinnamomum verum*), Tumeric (*Curcuma longa*) and ginger (*Zingiber officinale)* on broiler chickens, evaluated by the expression profiles of core proinflammatory (IL-1β, IL-6, TNF-α, IFN-γ) and anti-inflammatory (IL-4, IL-10, TGF-β) genes.

The specific objectives will be to

1. standardize and validate the extraction protocols for aqueous extracts of the selected phytogenics,
2. assess the impact of phytogenic extracts on growth and carcass characteristics of two strains of broiler chickens,
3. assess the effect of phytogenic treatments on the blood profile and immune biomarkers of the chickens,
4. establish dose-response relationships and optimal administration strategies for each extract or their synergistic combinations in two strains of broiler chickens,
5. characterize the changes in mRNA expression levels of selected proinflammatory (IL-1β, IL-6, TNF-α, IFN-γ) and anti-inflammatory (IL-4, IL-10, TGF-β) cytokine genes in broiler lymphoid and caecal tissues following phytogenic supplementation.
6. appraise the potential of optimized phytogenic supplementation as a replacement for antibiotics in the Nigerian poultry systems and inform industry recommendations and regulatory policies; and
7. develop an organic growth promoter and immune booster (Nasboost®) for broiler chickens in Nigeria.

Research Hypothesis

Null Hypothesis (H_0): Phytogenic aqueous extracts will have no significant effect on the mRNA expression levels of pro-inflammatory and anti-inflammatory genes in the spleen and cecal tonsils of two strains of broiler chickens.

Alternative Hypothesis (H_A): Phytogenic aqueous extracts will significantly modulate the immune response by:

H_Aa: Significantly downregulating the mRNA expression of pro-inflammatory genes.

H_Ab: Significantly upregulating the mRNA expression of anti-inflammatory genes.

H_AC: A combination of Phytogenic aqueous extracts will produce a synergistic effect, resulting in a more pronounced immunomodulatory response than when used singly.

Literature review

Phytogenic additives and extracts

Phytogenics may be classified based on therapeutic functionality, active ingredients, or application characteristics (Mobasher, 2025). Phytogenic substances are a group of natural herbal growth promoters or non-antibiotic growth promoters used as feed additives, derived from herbs, spices, or other plants. They have biologically variable activities and are thought to possess certain growth-promoting effects like antibiotics (Rossie *et al*., 2020). Various herbs along with their products have been validated to be beneficial due to anti-microbial, anti-oxidant, anti-inflammation, and immunomodulation properties, with no negative effects on growth and feed efficiency. (Kuralakar and Kuralakar, 2021). There is a growing tendency to use natural herbal products due to their lack of negative effects. The emergence and use of phytogenic additives have gained attention in animal nutrition research and are important for livestock husbandry and product quality.

Phytogenic extracts contain bioactive compounds with the potential to improve livestock health, growth performance, reproduction, product quality, and to reduce emissions and toxicity. They are preferred due to their economic affordability, ease of accessibility, and preparation. In addition, they generate less residual mass and have no toxic effects on livestock productivity; hence, they are safe for human beings. Antimicrobial activity, immune modulatory activity, antioxidant property, and intestinal microbiota regulation are the known modes of action of phytogenic feed additives. Using tools related to metagenomics, transcriptomics, proteomics, and network pharmacology may illuminate the functions and acting modes of these additives and establish some inexpensive methods to practice these additives as alternatives for antibiotics in livestock production systems.

They can be fed to animals through dietary supplementation in dried, crushed, or extract form, or they can be offered to animals as green fodders. Moreover, multiple herbal products or extracts can be used in combination for multi-

beneficial effects. They exert an integrated influence through synergism among their bioactive components (Albino and Arma, 2022). Various research has been conducted to look at blends of bioactive components as compared to a single compound; however, the significance of an individual ingredient and or bioactive component cannot be underestimated.

Phytogenic compounds exert strong antimicrobial effects by disrupting bacterial cell membranes, interfering with quorum sensing, and inhibiting the activity of enzymes critical for pathogen survival, particularly targeting Gram-positive bacteria such as Clostridium perfringens, Escherichia coli, and Salmonella *spp* (Mahfuz *et al*. 2021). Notably, essential oils such as thymol and carvacrol destabilize membrane integrity and metabolic processes in harmful microbes.

Antioxidant activities arise from polyphenols, flavonoids, and terpenes, which neutralize reactive oxygen species (ROS), upregulate endogenous antioxidant enzymes (SOD, CAT, GPx), and protect tissues from oxidative injury, crucial in the high-metabolic broiler systems (Mobasher, 2025).

Herbal extracts of *Cinnamomum verum*, *Allium sativum*, *Zingiber officinale* and *Origanum vulgare* significantly boosted gain in body weight and FCR in turkey and quails (Gernat *et al*., 2021). Feeding peppermint leaves to laying hen improved egg number and weight.

Proinflammatory and anti-inflammatory cytokine genes

The categorization of cytokines as either proinflammatory or anti-inflammatory is fundamental for understanding their roles in immune regulation, disease resistance, and homeostasis (Krzysica *et al*., 2022). The molecular tapestry of proinflammatory and anti-inflammatory gene networks in chickens is foundational to the avian immune system's capacity to respond to, control, and recover from infectious challenges. Proinflammatory genes—including IL-1β, IL-6, TNF-α, IFN-γ, and IL-8—initiate, amplify, and resolve immune responses, while anti-inflammatory genes—IL-10, IL-4, and TGF-β—temper inflammation and promote tissue healing (Figure 1). The balance between these gene products is dynamically modulated by the genetic makeup of the host, environmental pressures, pathogen load, and nutritional status. The regulation, expression dynamics, and genetic variation of these genes determine individual and breed resilience to pathogens. In addition, the integration of cytokine gene data into systems biology and precision breeding will continue to unlock new opportunities for avian health, welfare, and productivity, ultimately contributing to food security and the global poultry sector's resilience.

Proinflammatory cytokines are principal coordinators of host defense, initiating and amplifying inflammatory responses essential for pathogen clearance and the

activation of adaptive immunity. Produced predominantly by myeloid cells (macrophages, dendritic cells, monocytes), T-lymphocytes, and epithelial cells, these cytokines modulate a range of cellular activities, including cell proliferation, differentiation, migration, and survival. In chickens, the rapid engagement of proinflammatory signaling provides the critical early defense against a range of viral, bacterial, and protozoal pathogens (Hamdy, 2021).

The principal proinflammatory genes in chickens encode for cytokines such as IL-1β, IL-6, TNF-α, IFN-γ, and IL-8. These mediators not only stimulate cell-autonomous antimicrobial programs, chemotaxis, and immune effector functions but also play a pathophysiological role in tissue injury if their production is excessive or uncontrolled. The expression of these genes is tightly regulated at transcriptional, post-transcriptional, and epigenetic levels, and is dynamically modulated in response to infection, stress, nutrition, and genetic background (Xin *et al*., 2018).

To maintain homeostasis and prevent the detrimental effects of unchecked inflammation, chickens express a suite of anti-inflammatory genes encoding cytokines and regulatory proteins. Cytokines such as IL-10, IL-4, and TGF-β act in concert to suppress proinflammatory signaling, support resolution, and promote tissue repair or immune tolerance (Chaudhari *et al*. 2018). The ability to mount an effective anti-inflammatory response is equally as crucial as proinflammatory defense, as overactive inflammation is a principal cause of tissue damage, autoimmunity, and compromised productivity in commercial poultry operations (Meuinier *et al*. 2025).

Cytokines as markers for disease resistance in poultry

Genome-wide association studies and selective breeding are increasingly utilizing cytokine gene expression profiles as reliable markers for disease resistance against key poultry pathogens such as Salmonella, Eimeria, NDV, and Marek's disease virus (Kumar *et al*. 2010). Gul *et al*. (2022) and Meunier *et al*. (2025) reported that enhanced expression of IL-6, IL-8, and IFN-γ correlates with resistance to enteric infections, while elevated anti-inflammatory IL-10 may, paradoxically, impede pathogen clearance and reduce vaccine efficacy when unchecked. Unbalanced proinflammatory and anti-inflammatory responses are linked not only to immune-mediated pathologies (sepsis, amyloidosis, immunosuppression) but also to production losses such as poor growth, inefficient feed conversion, and inferior meat/egg quality. Chronic stressors (e.g., heat, oxidative, dietary) also alter cytokine expression, impacting both animal welfare and economic outputs (Goel *et al*., 2021).

Effects of Phytogenics on Immune Function

Phytogenic feed additives have repeatedly been shown to enhance both innate and adaptive immune responses. Several phytogenic compounds modulate immune responses by affecting cytokine gene expression (downregulation of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α; and upregulation of anti-inflammatory mediators), promoting proliferation and differentiation of lymphocytes, improving antibody titers post-vaccination, and supporting mucosal immunity through increased secretory IgA (SIgA) levels (Ceylan *et al*., 2025). Engida *et al*. (2023) reported increased antibody titers against infectious bursal disease (IBD) vaccine in broiler trials with rosemary and thyme supplementation. Ceylon *et al*. (2025) reported Cytokine regulation following downregulation of pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α) and upregulation of anti-inflammatory IL-10 following PFA supplementation. The authors also observed elevated counts of CD4+ and CD8+ T lymphocytes (stimulation of lymphocytes) in the intestines of broilers fed phytogenic blends, with a dose-dependent response evident for blends containing Ginkgo biloba and Silybum marianum. Enhanced mucosal and systemic immunity was observed with increased SIgA, IgG, IgM, and resistance to viral or bacterial challenge, especially when the challenge is concurrent with environmental or nutritional stress. However, Phillips *et al*. (2025) noted that excessively high doses or certain combinations may reduce immunocompetence, emphasizing the importance of dose determination and blend optimization.

Impact of Phytogenics on Gut Health and Microbiota

The gastrointestinal health of broilers is a primary determinant of productivity, resilience, and food safety. One of the most consistent effects of PFAs is the selective modulation of gastrointestinal microbiota: inhibition of pathogenic bacteria (e.g., E. coli, Salmonella) and the promotion of beneficial lactic acid bacteria (e.g., Lactobacillus, Bifidobacterium). This shift in microbial populations underpins many of the observed benefits for gut health, immunity, and productivity (Enigida *et al*., 2025). PFAs affect gut integrity, morphology, and microbial communities by improving villus height and crypt architecture, resulting in increased absorptive area and enhanced nutrient assimilation (Paraskeuas and Mountzouris, 2019). Mobasher (2025) also reported that PFAs stimulate digestive enzymes and mucus secretion, bolstering the gut's barrier function and reducing pathogen binding to the epithelium. In addition, they culture-based and molecular studies in broilers supplemented with a variety of promote beneficial microbiota (Lactobacilli, Enterococci), evident from both PFAs including basil, rosemary, thyme, lemon grass, and peppermint (Paraskeuas and Mountzouris, 2019). Suppression of pathogenic bacteria including E. coli and Clostridium perfringens, leads to reduced enteric pathogen load and improved resilience to intestinal infections (Eniga *et al*., 2023).

Effects of Phytogenics on Growth Performance

Several studies have demonstrated that PFAs can improve weight gain, average daily gain (ADG), feed intake (FI), and feed conversion ratio (FCR) compared to controls, with some effects equivalent or superior to antibiotic growth promoters (AGPs) under both optimal and challenging rearing conditions (Michalczuk *et al*. 2024; Gopakumar *et al*., 2025; Enigida *et al*., 2023). Blends of thymol and carvacrol, cinnamon oil, or oregano oil have been shown to consistently increase body weight and decrease FCR by 2–10% across diverse experimental models. (Mahfuz *et al*., Michalczuk *et al*., 2024, Gopakumar *et al*., 2025). Enigida *et al*. (2023) reported that using blends of basil, rosemary, lemongrass, peppermint, and thyme found that lemongrass and thyme supported the highest overall BWG and ADG, with improved FCR, while peppermint and rosemary had variable effects depending on dose and phase. Dietary supplementation of nano-encapsulated curcumin, garlic, and green tea extracts further improved growth performance, indicating that advances in delivery technologies can boost efficacy (Obianwuna *et al*., 2024). Though most reports confirm growth-promoting effects, results sometimes vary with formulation, botanical source, concentration, feed matrix, and rearing environment, highlighting the need for standardization and quality control.

Emerging fields like nutrigenomics employ RNA-Seq, microarrays, and qRT-PCR to study how dietary interventions modulate cytokine gene expression, impacting immune competence and productivity (Girija *et al*., 2025). Feeding trials reveal that nutrients such as vitamin E, selenium, probiotics, and specific amino acids (e.g., L-arginine) can up- or down-regulate cytokine genes, enhancing disease resilience or mitigating inflammatory damage. Khatun *et al*. (2020) reported that Vitamin E supplementation was shown to downregulate TNF-α and IFN-γ while upregulating TGF-β1, contributing to reduced oxidative stress and improved immune function.

Effects of Phytogenics on Meat Quality

Besides productivity and health, meat quality and shelf life are major targets for innovation in broiler nutrition. Bokko *et al*. (2016) and Michalczuk *et al*. (2024) reported that phytogenic additives impact meat quality through reduction of lipid peroxidation and improved oxidative stability, leading to less drip loss, better color, reduced off-odors, and extended shelf life, particularly for breast and thigh meat stored under refrigeration or freezing. These authors also reported that PFAs rich in polyphenols, such as grape seed extract, rosemary, oregano, and chestnut extract, increased monounsaturated and polyunsaturated fatty acids (MUFAs, PUFAs), while reducing saturated fat in broiler meat. The PFAs also enhance meat tenderness and sensory characteristics by reducing muscle fibre area and increasing antioxidant content, with some also reducing the incidence of

myopathies such as wooden breast syndrome. Essential oils and PFAs reduce the proliferation of spoilage organisms and delay rancidity, producing meat more aligned with consumer quality expectations (Michalczuk *et al*., 2024).

Theoretical Framework

This study is grounded in the principles of nutritional immunomodulation. The central theoretical premise is that bioactive compounds within the phytogenics act as signaling molecules that can influence intracellular inflammatory pathways, primarily the Nuclear Factor-kappa B (NF-κB) pathway. In a state of stress or infection, the NF-κB pathway is activated, leading to the transcription and subsequent upregulation of pro-inflammatory cytokine genes like TNF-α, IL-1β, and IL-6. These cytokines orchestrate the inflammatory response. The hypothesis is that the phytochemicals in ginger and garlic can inhibit the activation of NF-κB. This inhibition would lead to the downregulation of pro-inflammatory gene expression. Concurrently, these compounds may promote pathways that lead to the upregulation of anti-inflammatory cytokines, such as IL-10 and TGF-β, which are crucial for resolving inflammation and returning the system to homeostasis. By measuring the mRNA levels of these specific genes, we can directly test this framework and visualize the "re-balancing" of the immune response.

Methodology

Study Site

The research will be conducted at the Poultry Unit of the University of Maiduguri Livestock Teaching and Research Farm, and the North East Biotechnology Centre, Maiduguri, Borno State. Maiduguri is located on Latitude 11^{o} 5’ N and Longitude 30^{o} 09’ E and an altitude of 354 m above sea level in the North-Eastern part of Nigeria. The temperature of the area ranges from 24 to 40 °C. The annual rainfall of Maiduguri averages 552.1mm (21.7) per year. The ecological zone of Borno is characterized by vast grassland and few trees.

Experimental Animals and Designs

One thousand four hundred (1400), day-old broiler chicks of two strains (Cobb 500 and Ross 308) will be obtained from a reputable hatchery and used for the study in four Experiments. They will be reared at the Poultry unit of the University of Maiduguri Livestock Teaching and Research farm. The phytogenics will be grouped into two; botanicals (bitter leaf, neem leaf, Moringa leaf, eucalyptus leaf, lemon grass and scent leaf) and herbs (cinnamon, clove, garlic, ginger and tumeric). For experiment 1, 350 birds (175 each of Cobb 500 and Ross 308 strains) will be randomly allotted to treatments - (T1; control, T2; bitter leaf, T3; Neem leaf, T4; Moringa leaf, T5; eucalyptus leaf, T6; Scent leaf, and T7; lemon grass). Each treatment will consist of 25 birds/strain in five replicates of 5 birds each. Experiment 2 will have the herbs allotted to treatments thus; T1; control, T2; clove, T3; cinnamon, T4; garlic, T5; ginger, and T7; turmeric. Experiment 3 will have 2 or more combinations of the botanicals for each treatment, while Experiment 4 will be a combination of the herbs for each

treatment. Each experiment will last for 8 weeks and will involve administering the phytogenics in water as an aqueous extract. Birds will be fed *ad libitum* throughout the study. Aqueous extract of the test materials will be provided in the morning, and clean drinking water later. Birds will be raised under the same conditions of humidity, ventilation, and temperature.

Preparation of the aqueous extract

The phytogenics (botanicals and herbs) will be sourced fresh from reputable sources, authenticated by botanists, and processed rapidly to retain bioactivity. Dried (or fresh) leaves/roots will be macerated in distilled water at a 1:10 (w/v) ratio, agitated for 12–24 hours at room temperature, then filtered using sterile muslin/Whatman filter paper. The filtrate will be concentrated under reduced pressure (rotary evaporator) and stored at 4°C for short-term use. Extract concentration will be measured (mg/mL), and phytochemical profiling (total phenolics, alkaloids, saponins, flavonoids, tannins) is conducted via spectrophotometric and chromatographic (HPLC) methods. Extracts will be screened for contaminants, mycotoxins, and microbial load according to NAFDAC standards for feed additives. Extracts will be added to drinking water at standardized concentrations for 42 days

Sample Collection

In weeks 4, 6, and 8 of each experiment, two birds per replicate (10 birds per treatment) will be randomly selected and humanely euthanized. cecal tonsil tissues will be aseptically harvested, immediately immersed in 2 ml of RNA Shield in a sample bottle, and stored at -20°C for gene expression analysis.

Total RNA extraction and reverse transcription

Approximately 30 mg of caecal tissue sample will be submerged in RNA Shield® (Sigma-Aldrich, USA) and homogenised in 500 uL QIAzol lysis reagent for 10 min at 30 Hz in a Tissuelyzer LT (Qiagen, UK). Lysates will be mixed with 100 µL chloroform, transferred to peg Gold Phase Trap tubes (PeqLab, UK), and centrifuged for 5 mins at room temperature. The aqueous phase will then be poured into fresh tubes, mixed with 1.5 volumes of ethanol, and applied to Qiagen RNeasy columns (Qiagen, UK). RNA will be purified according to the manufacturer's instructions (Qiagen, UK). RNA integrity will be assessed using an Agilent Bioanalyzer. Purity and quantity will be measured using a NanoDrop spectrophotometer; for all samples, the absorbance peak will be at 260 nm, A260/280 > 2, and A260/230 > 1. 800 ng of RNA will be reverse transcribed using a reverse transcription kit (Qiagen, UK) in a 10 µL reaction according to the manufacturer's instructions. This RT kit will include a mandatory gDNA wipe out step. The completed reaction will be diluted 10-fold with 5 µg/mL tRNA in water.

Quantitative real-time PCR (qPCR)

Two microliters of cDNA will be amplified in a 10 μL reaction using Agilent Brilliant III SYBR Ultra-Fast SYBR Green mix, with each primer at a final concentration of 500 nmol/L. The no-template control reaction will contain 2 μL of tRNA (0.5 μg/mL). Amplification parameters will be: 95°C for 3 minutes, followed by 40 cycles of 95°C for 5 seconds and 57°C for 1 second in a thermocycler. Melt curves will be checked for product specificity (single peak) and the presence of primer dimers. All primers will be designed to be intron-spanning so that any residual gDNA cannot be detected, and to avoid known SNPs and secondary structures. Assays will be designed and tested for specificity through electrophoresis, with an efficiency >95%, sensitivity to 10 copies per reaction, and linearity over 7 logs by qPCR. Copy numbers per reaction will be derived from standard curves using Rotor-Gene software. Primers (forward and reverse) will be designed for each proinflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-4, IL-10, TGF-β) gene using NCBI/Ensembl chicken sequences, validated for efficiency (90–110%), specificity (via melt-curve and electrophoresis), and absence of dimers. Reference genes (housekeeping genes) will be selected based on their stability in chicken immune tissues using geNorm software. Cycle threshold (Ct) values for each sample will be recorded, and the average Ct values from duplicate qPCR reactions will be calculated. The relative gene expression at different chicken ages will be determined using the comparative Ct method: 2-ΔΔCt (Livak and Schmittgen, 2001). The differential gene expression between strains and treatments will be statistically analyzed using the Wilcoxon test.

Data Collection

Growth performance, meat quality, and carcass profiles

Data will be collected on growth performance traits such as body weight, feed consumption, weight gain, feed conversion ratio, and mortality on a weekly basis up till eight weeks of age for each experiment. Body weight will be measured per cage on arrival, as well as weekly up until the day of slaughter at eight weeks of age, using a sensitive weighing scale. Weekly feed intake will be calculated according to the total feed consumed per week per cage up until the day of slaughter. Thereafter, the average feed intake per bird per week will be determined, and Mortalities recorded. Live weight gain, ADG, and FCR will be calculated.

At the end of the 8-week feeding trial,10 birds from each strain per treatment (2 from each replicate) will be randomly selected, fasted, slaughtered, defeathered, and eviscerated. Carcass characteristics will include: dressing percentage, thigh, breast, drumstick, and carcass weights. Organ weights such as heart, spleen, intestine, gizzard, and caecum. Organs' percent to live weight and percent of carcass parts to carcass weight will be determined.

Statistical Analysis

Data generated from growth performance; body weight, weight gain, feed intake, feed conversion ratio, and weight and percentage of individual carcass components, as well as weight and percentage of viscera organs, will be analysed and presented as means ± standard error of means. All parameters will be compared among the chicken genotypes using a two-way analysis, followed by a a post hoc test. Significant means will be separated using Duncan's Multiple Range Test. The analyses will be carried out using SAS Statistical software. The following statistical model will be applied:

$Y_{ijk} = \mu + A_i + B_j + C_k + e_{ijkl}$

Where,

Y_{ijkl} = information belonging to all observations;

μ = the population mean;

A_i = effect of strain (1, 2)

B_j= fixed effect of phytogenics (1, 2)

C_k= effect of Strain x phytogenic interaction,

e_{ijkl} = residual error.

Expected outcome

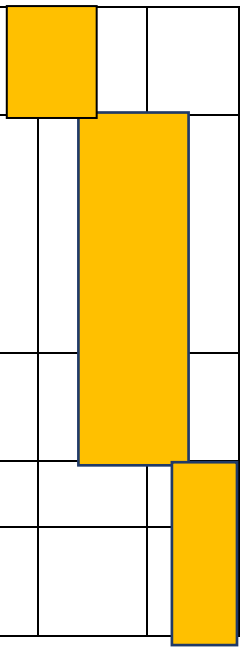
- This research will provide molecular-level evidence to support the use of phytogenics as effective immunomodulators and growth promoters, and sustainable alternatives to antibiotics in broiler production, thereby increasing the profitability and sustainability of small and medium-scale poultry enterprises and contribute to reduction in protein and food insecurity and poverty in Nigeria.
- The production of healthier, antibiotic-free poultry products for consumers directly combats the development of AMR and safeguard the efficacy of life-saving drugs for human medicine.
- The global phytogenic feed additives market, valued at $1.8 billion in 2023, is projected to reach $2.8 billion by 2030, presenting significant commercialization opportunities.
- It will lead to a reduction in mortality and morbidity, thus improving poultry productivity.
- Establishment of optimal dosing regimens for maximum immunomodulatory benefit while minimizing potential adverse effects or production costs.
- Comprehensive molecular data will support regulatory approval processes for phytogenic feed additives in various international markets.

- Enhanced broiler production efficiency and disease resistance will contribute to sustainable protein production for growing global populations.
- This research will add value to Nigerian plant resources and create economic opportunities for rural communities while contributing to agricultural diversification.
- Finally, standardized natural phytogenic products with scientifically validated immune-enhancing and growth-promoting properties for broiler production will be developed for commercial use. Thus, this research is poised to deliver transformational impact for industry stakeholders, regulatory authorities, and the global effort to safeguard public health, animal welfare, and agricultural sustainability.

Time Frame: Timeline for the major activities of the project

S/N	Description of Activity	Duration	Year	Quarter			
				1st	2nd	3rd	4th
1.	Procurement of equipment and consumables	1 month	2026				
2.	Procurement of equipment and consumables	I month	2026				
3.	Procurement and rearing of broiler chickens for Experiment 1	1 month	2026				
4.	End of experiment 1, data and blood samples collection	1 month	2026				
5.	RNA Extraction and cDNA Synthesis and expression profiling, Procurement and rearing of broiler chickens for Experiment 2	1 month	2026				
6.	End of experiment 2, data and blood samples collection	1 month	2026				
7.	RNA Extraction and cDNA Synthesis and expression profiling, Procurement and rearing of broiler chickens for Experiment 3	1 month	2026				

8.	End of experiment 3, data and blood samples collection	1 month	2026				
9.	RNA Extraction and cDNA Synthesis and expression profiling, Procurement and rearing of broiler chickens for Experiment 4	1 months	2026				
10	End of experiment 4, data and blood samples collection	1 months	2026				
11	Data analyses	1 month	2026				
12	Report writing and Submission	1 month	2026				



Budget

DESCRIPTION OF ITEM	Quantity	Unit cost	Total
1. Personnel Costs/Allowances			
Principal Investigator	1	160000	1920000
Team Members	3	100000	3600000
Technical Support	2	40000	960000
Others (Mentees)	2	20000	480000
Sub-Total			**6,960,000.00**
2. Equipment (List & Specify)			
dryer	1	550000	550000
Evaporator	1	340000	340000
Precision digital scale (100x0.01g)	2	65000	130000
Solar system (5kva)	1	3000000	3000000
Electric/gas brooder	2	450000	900000
feeder, drinkers, brooder guards	1	500000	500,000
Sub-Total			**5,420,000.00**
3. Supplies/Consumables			
Day-old broiler chickens	1400	2000	2800000
Poultry feed	280	26000	7280000
Renovation of Poultry House	1	2500000	2500000
Phytogenics	11	40000	440000
RNA kit (250 Preps)	5	500000	2500000
One-step RT-PCR kit	5	600000	3000000
qPCR tubes	1000	600	600000
Pipettes	10	90000	900000
pipette tips	30	15000	450000
Nuclease-free Eppendorf tubes	10	50000	500000

RNA Zap	5	300000	1500000
RNA shield	1	700000	700000
Primers	10	50000	500000
Latex gloves	5	30000	150000
wipes	10	7000	70000
Genomic Lysis Buffer (50 ml)			65000
Agarose 100g			58000
Boric acid (99.5%) 250g			25000
Sodium citrate, anhydrous (98.0%)			30000
Basic Research plus 0.5-10ul			150000
Basic Research plus 10-100Ul			150000
Micro Tube Rack for 1.5 and 2.0-mL tubes, 36 wells (3 rows of 12 wells each),			50000
Absolute Alcohol (Non-DNA)			40000
Blood profile determination			1000000
phytochemical Screening			500000
Vaccines			100000
Subtotal			
4. Data Collection & Analysis			
Data collection, entry, and coding			200000
statistical Analysis			200000
Software (Office packages, antivirus, Data management)			200000
Project Secretariat (stationery/Printer)			900000
HP Laptop co*i*7			950000
Sub Total			**2,450,000**
5. Travels			
Distribution of products for testing			500000
Sub-Total			**500,000**
6. Dissemination			
Journal Publications			600000
Conference attendance			2000000
Sub-Total			**2,600,000**
Contingency (10%)			**4398800**
GRAND TOTAL			**48,386,800.00**

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