UNIVERSITI PUTRA MALAYSIA

Piper betle L. AND Persicaria odorata (Lour.) Soják AS ALTERNATIVE TO ANTIMICROBIAL GROWTH PROMOTERS IN BROILER CHICKENS

ABDUL BASIT MUHAMMAD

FPV 2022 15

***Piper betle* L. AND *Persicaria odorata* (Lour.) Soják AS ALTERNATIVE TO ANTIMICROBIAL GROWTH PROMOTERS IN BROILER CHICKENS**

By

ABDUL BASIT MUHAMMAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

November 2021

DEDICATION

This work is dedicated to the souls of my late father and mother (May Allah rest their soul in the peace and grant them higher ranks in Jannah) My beloved wife, son, and daughter for being my inspiration, their unconditional support made it easier for me to complete the tedious journey of PhD My brother and sisters for their constant encouragement and support My friends and colleagues.

Abdul Basit Muhammad
November 2021

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairperson : Assoc. Prof. Arifah binti Abdul Kadir, PhD
Faculty : Veterinary Medicine

In several countries, except Europe, dietary supplementation of sub-therapeutic antimicrobial growth promoters (AGPs) is commonly practised for sustainable broiler chicken production. However, their indiscriminate use resulted in antibiotic resistance, which leads to a ban against AGPs. Hence, it is essential to find potential and safe alternatives that can improve broiler chickens' production performance. Recently, phytobiotics, especially herbs, have gained attention and have been extensively studied for their possible use as an alternative poultry feed additive. Generally, phytobiotics are assumed to be natural, safe, and residue-free substances; however, scarce literature is available about their optimal dosage and safe use. The present study was carried out to assess the phytochemical screening, quantification of secondary metabolites, acute oral toxicity, appropriate dosage and potential of selected herbs *Piper betle* and *Persicaria odorata* as alternative growth promoters to AGPs in broiler chickens.

P. betle and *P. odorata* leaf extracts were evaluated for qualitative and quantitative phytochemical screening, antioxidant and antimicrobial potential. The preliminary phytochemical analysis of *P. betle* and *P. odorata* leaf extracts revealed the presence of flavonoids, phenols, saponins, tannins, glycosides, and volatile oils. The higher total phenolic content and total tannins were quantified from *P. betle* methanolic leaf extract. Additionally, it showed increased antioxidant activity compared to *P. odorata* leaf extracts. The in *vitro* antibacterial potential of both herbs was estimated against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica, Pseudomonas aeruginosa, Candida albicans,* and *Aspergillus brasiliensis.* The methanolic leaf extract of *Piper betle* showed antibacterial and antifungal activity against selected strains. In conclusion, phytochemical screening of *P. betle* and *P. odorata* leaf extracts showed rich diversity of phytochemicals, antioxidant, and antimicrobial activity.

In the first phase, quantitative phytochemical analyses of methanolic extract of *P. betle* and *P. odorata* was carried out using chromatography. Furthermore, the limit dose acute toxicity of the selected herbs was estimated in broiler chickens according to the guidelines of OECD, 2016. A total of 35 broiler chicks were used in this study. On day 21 of age, the chicks were randomly allocated into 7 treatment groups. The birds in extract-treated groups were orally gavaged with a single dose of methanolic leaf extract of *P. betle* and *P. odorata* at the rate of 500mg/kg, 1000 mg/kg, and 2,000 mg/kg, respectively. At the same time, the control group received 0.5% carboxymethyl cellulose (CMC) as a placebo. The gas chromatography-mass spectrometry (GC-MS) fingerprints showed the presence of various metabolites. Furthermore, the secondary bioactive compounds eugenol and quercetin were successfully quantified from *P. betle* and *P. odorata,* respectively. Acute toxicity study has shown no behavioural or physical changes in the chickens of treated groups. There were no significant differences in growth performance, haematological indices, serum biochemistry, and relative internal organs weights between the control and extract treated groups. The gross and microscopic observations indicated no changes in the liver, heart, lungs, spleen, gizzard, and kidneys of the treated birds. In conclusion, secondary bioactive compounds eugenol and quercetin were successfully quantified from methanolic leaf extract of *P. betle* and *P. odorata*. Based on current study results, the LD_{50} value for the tested extracts would be > 2000 mg/kg body weight.

The second phase was conducted to estimate the influence of different dose supplementation of *Piper betle* leaf meal (PBLM) and *Persicaria odorata* leaf meal (POLM) on growth performance, ileal digestibility, gut morphology, haematological indices, serum biochemical attributes, histomorphology of the liver, and internal organs parameters in broiler chickens. A total of 210 day-old broiler chicks were fed either basal diet (BD) or BD + 2, 4, and 8 g/kg of PBLM and POLM. Except for PBLM 8g/kg, graded dose inclusion of PBLM and POLM increased ($p < 0.05$), the body weight gain (BWG) positively modulated the gut architecture and enhanced nutrient digestibility in broiler chickens. Birds fed on PBLM 4g/kg and POLM 8g/kg had significantly higher ($p < 0.05$) BWG with superior ($p < 0.05$) feed efficiency. Haematological indicators like red blood cells and haemoglobin were positively improved in birds fed on diet PBLM 4g/kg and POLM 8g/kg compared to the control group. Additionally, dietary supplementation of PBLM and POLM decreased the activity of AST and ALT with reduced serum concentration of triglyceride and cholesterol, which were observed lowest in PBLM 4g/kg and POLM 8g/kg groups. On the other hand, the serum concentrations of total protein (TP), albumin, and globulin were recorded as increased ($p < 0.05$) in broilers raised on the diets PBLM 4g/kg and POLM 8g/kg. Notably, dietary supplementation of PBLM and POLM did not influence the organ parameters and showed no adverse effects on liver histomorphology. In conclusion, dietary supplementations of phytobiotics (PBLM 4 g/kg and POLM 8 g/kg) positively modulated the intestinal microarchitecture with enhanced nutrient digestibility. Hence, it resulted in maximum body weight gain with improved haematological indicators and serum biochemistry attributes. Furthermore, dietary supplementations of phytobiotics have no deleterious effects

on the internal organs and retained relatively normal hepatic parenchyma, thus enhanced the optimum growth performance of the broiler chickens.

The third phase experiment was designed to estimate the comparative efficacy of PBLM and POLM with AGPs on growth performance, gut morphology, ileal digestibility, caecal microbiota composition, tibial bone morphometry, and meat quality in broilers chickens. Based on the earlier trial results, PBLM 4g/kg and POLM 8g/kg were selected for the comparative efficacy study. A total of 150 day-old broiler chickens were fed basal diet (BD), which served as the negative control (NC) or BD + 0.2g/kg tetracycline as positive control (PC); BD + 0.03g/kg halquinol (HAL), BD + 8g/kg POLM (Po8), and BD + 4g/kg PBLM (Pb4) as treatment groups. Dietary supplementation of phytobiotics Po8 and Pb4 showed improved growth performance, positively modulated the gut architecture, and improved digestibility comparable to AGPs. Additionally, dietary supplementation of phytobiotics (Po8 and Pb4) significantly decreased the *E. coli*, *Salmonella*, and *Staphylococcus aureus* compared to the NC group. However, supplementation of Pb4 resulted in significantly decreased total anaerobic bacteria and Clostridium counts compared to the NC group. Importantly, dietary supplementation of phytobiotics significantly increased the *Lactobacillus* count compared to HAL, PC, and the NC groups. The supplementation of phytobiotics improved meat quality compared to the NC group. Moreover, significantly ($p < 0.05$) higher values of geometric parameters of tibial bones with increased strength were recorded in chickens fed on the phytobiotics, compared to the NC group. Conclusively, phytobiotics showed performance comparable to the selected AGPs. The present study results provided evidence that the dietary supplementation of PBLM 4 g/kg and POLM 8 g/kg are the appropriate and safe doses. PBLM and POLM could be the potential alternatives to AGPs for sustainable broiler chicken production.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

Piper betle L. DAN _Persicaria odorata_ (Lour.) Soják SEBAGAI ALTERNATIF TERHADAP PENGGALAK PERTUMBUHAN ANTIMIKROB PADA AYAM PEDAGING

Oleh

ABDUL BASIT MUHAMMAD

November 2021

Pengerusi: **Profesor Madya Arifah binti Abdul Kadir, PhD**
Fakulti : **Perubatan Veterinar**

Di beberapa negara, kecuali Eropah, suplemen makanan sub-terapi penggalak pertumbuhan antimikroba (AGP) biasanya dilakukan untuk pengeluaran ayam pedaging yang lestari. Walau bagaimanapun, penggunaan sembarangan menyebabkan rintangan terhadap antibiotik yang menyebabkan larangan terhadap penggunaan AGP. Oleh itu, adalah mustahak untuk mencari alternatif yang berpotensi dan selamat yang dapat meningkatkan prestasi pengeluaran ayam pedaging. Baru-baru ini, fitobiotik, terutama herba, mendapat perhatian dan telah banyak dikaji untuk kemungkinan penggunaannya sebagai bahan tambahan makanan ternakan. Secara amnya, fitobiotik dianggap sebagai bahan semula jadi, selamat, dan bebas residu; namun, terdapat sedikit maklumat mengenai dos yang optimum dan penggunaan yang selamat. Kajian ini dijalankan untuk menaksir saringan fitokimia, pengkuantitian metabolit sekunder, ketoksikan oral akut, dos dan potensi yang sesuai dari herba terpilih *Piper betle* dan *Persicaria odorata* sebagai penggalak pertumbuhan alternatif terhadap penggalak pertumbuhan antimikrob pada ayam pedaging.

Pada mulanya, ekstrak daun *P. betle* dan *P. odorata* dinilai untuk saringan fitokimia kualitatif dan kuantitatif, potensi antioksidan dan antimikrob. Analisis fitokimia awal ekstrak daun *P. betle* dan *P. odorata* mendedahkan adanya flavonoid, fenol, saponin, tanin, glikosida, dan minyak mudah meruap. Kandungan fenolik total dan jumlah tanin yang lebih tinggi dikuantifikasi dari ekstrak metanol daun *P. betle*. Selain itu, ia menunjukkan peningkatan aktiviti antioksidan berbanding dengan ekstrak daun *P. odorata*. Potensi *in vitro* antibakteria kedua-dua ramuan tersebut dianggarkan terhadap *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Candida albicans*, dan *Aspergillus brasiliensis*. Ekstrak metanol dari daun *P. betle* menunjukkan aktiviti antibakteria

dan antikulat terhadap jenis-jenis terpilih ini. Kesimpulannya, saringan fitokimia ekstrak daun *P. betle dan P. odorata* menunjukkan fitokimia kepelbagaian beraneka, aktiviti antioksidan dan antimikrob.

Pada fasa pertama, analisis kuantitatif fitokimia ekstrak metanol *P. betle* dan *P. odorata* dilakukan dengan menggunakan kromatografi. Selanjutnya, ketoksikan akut dos had ramuan herba terpilih dianggarkan pada ayam pedaging mengikut garis panduan OECD, 2016. Sebanyak 35 ekor ayam pedaging digunakan dalam kajian ini. Pada hari ke-21, anak-anak ayam tersebut diagihkan secara rawak ke dalam 7 kumpulan rawatan. Anak ayam dalam kumpulan yang dirawat ekstrak diberi oral dengan satu dos ekstrak metanol daun *P. betle* dan *P. odorata* pada kadar masing-masing 500mg / kg, 1000 mg / kg, dan 2000 mg / kg. Pada masa yang sama, kumpulan kawalan menerima 0.5% karboksimetil selulosa (CMC) sebagai plasebo. Gambar kromatografi-spektrometri massa (GC-MS) menunjukkan adanya pelbagai metabolit. Selanjutnya, sebatian bioaktif sekunder eugenol dan quercetin berjaya diukur masing-masing dari *P. betle* dan *P*. odorata. Kajian ketoksikan akut tidak menunjukkan perubahan tingkah laku atau fizikal pada kumpulan ayam yang dirawat. Tidak ada perbezaan yang signifikan dalam prestasi pertumbuhan, indeks hematologi, biokimia serum, dan berat organ dalaman relatif antara kumpulan kawalan dan ekstrak yang dirawat. Pemerhatian kasar dan mikroskopik menunjukkan tidak ada perubahan pada hati, jantung, paru-paru, limpa, hempedal, dan ginjal ayam yang dirawat. Kesimpulannya, sebatian bioaktif sekunder eugenol dan quercetin verjaya diukur dari ekstrak daun metanol *P. betle* dan *P. odorata*. Berdasarkan kepada keputusan kajian terkini, nilai LD50 untuk ekstrak *P. betle* dan *P. odorata* teruji ialah > 2000 mg / kg berat badan.

Fasa kedua dijalankan untuk menganggar pengaruh suplemen dos berbeza hidangan daun *P. betle* (PBLM) dan hidangan daun *P. odorata* (POLM) terhadap prestasi pertumbuhan, pencernaan ileal, morfologi usus, indeks hematologi, atribut biokimia serum, histomorfologi hati, dan parameter organ dalaman pada ayam pedaging. Sebanyak 210 ekor anak ayam pedaging diberi makan sama ada diet asas (BD) atau BD + 2, 4, dan 8 g / kg PBLM dan POLM. Namun untuk PBLM 8g / kg, kemasukan dos berperingkat PBLM dan POLM meningkat ($p < 0.05$), kenaikan berat badan (BWG), memodulasi positif arkitektur usus dan meningkatkan kecernaan nutrien pada ayam pedaging. Ayam yang diberi makan PBLM 4g / kg dan POLM 8g / kg secara signifikan lebih tinggi ($p < 0.05$) BWG dengan kecekapan makanan yang unggul ($p < 0.05$). Petunjuk hematologi seperti sel darah merah dan hemoglobin meningkat secara positif pada ayam yang diberi makan PBLM 4g / kg dan POLM 8g / kg berbanding dengan kumpulan kawalan. Selain itu, suplemen makanan PBLM dan POLM menurunkan aktiviti AST dan ALT dengan penurunan kepekatan serum trigliserida dan kolesterol, yang diperhatikan paling rendah dalam kumpulan PBLM 4g / kg dan POLM 8g / kg. Sebaliknya, kepekatan serum protein total (TP), albumin, dan globulin dicatatkan meningkat ($p < 0.05$) pada ayam pedaging yang diberi makan pada diet PBLM 4g / kg dan POLM 8g / kg. Terutamanya, suplemen makanan PBLM dan POLM tidak mempengaruhi parameter organ dan tidak menunjukkan kesan buruk pada histomorfologi hati. Kesimpulannya, makanan tambahan fitobiotik (PBLM 4 g / kg dan POLM 8 g / kg) secara positif memodulasi mikroarkitektur usus dengan peningkatan pencernaan nutrien. Oleh itu, ini menghasilkan kenaikan berat badan maksimum dengan penunjuk hematologi yang lebih baik dan atribut biokimia serum. Tambahan pula, makanan tambahan fitobiotik tidak mempunyai kesan buruk pada organ dalaman dan mengekalkan parenkima hepatik yang agak normal, sehingga meningkatkan prestasi pertumbuhan ayam yang optimum.

Eksperimen fasa ketiga dirancang untuk menganggar keberkesanan perbandingan PBLM dan POLM dengan AGP pada prestasi pertumbuhan, morfologi usus, pencernaan ileal, komposisi mikrobiota sekal, morfometri tulang tibial, dan kualiti daging pada ayam pedaging. Berdasarkan hasil percubaan sebelumnya, PBLM 4g / kg dan POLM 8g / kg dipilih untuk kajian perbandingan keberkesanan. Sebanyak 150 ekor ayam pedaging diberi makanan asas (BD), yang berfungsi sebagai kawalan negatif (NC) atau BD + 0.2g / kg tetrasiklin sebagai kawalan positif (PC); BD + 0.03g / kg halquinol (HAL), BD + 8g / kg POLM (Po8), dan BD + 4g / kg PBLM (Pb4) sebagai kumpulan rawatan. Suplemen diet fitobiotik Po8 dan Pb4 menunjukkan peningkatan prestasi pertumbuhan, modulasi positif arkitektur usus, dan peningkatan kecernaan yang setanding dengan AGP. Selain itu, suplemen diet fitobiotik (Po8 dan Pb4) menurunkan *E. coli*, *Salmonella*, dan *Staphylococcus aureus* secara signifikan berbanding kumpulan NC. Walau bagaimanapun, suplementasi Pb4 mengakibatkan penurunan jumlah bakteria anaerob secara signifikan, dan jumlah Clostridium dibandingkan dengan kumpulan NC. Yang penting, suplemen diet fitobiotik meningkatkan jumlah *Lactobacillus* secara signifikan berbanding kumpulan HAL, PC, dan NC. Tambahan fitobiotik meningkatkan kualiti daging berbanding dengan kumpulan NC. Selain itu, nilai parameter geometri tulang tibial yang lebih tinggi ($p < 0.05$) lebih tinggi dicatat pada ayam yang diberi makan fitobiotik, berbanding dengan kumpulan NC.

Kesimpulannya, fitobiotik menunjukkan prestasi yang setanding dengan AGP yang dipilih. Hasil kajian ini memberikan bukti bahawa suplemen makanan PBLM 4 g / kg dan POLM 8 g / kg adalah dos yang sesuai dan selamat. PBLM dan POLM boleh menjadi alternatif berpotensi untuk AGP untuk pengeluaran ayam pedaging yang lestari.

ACKNOWLEDGEMENTS

All praise belongs to ALLAH (SWT), the Beneficent, the Most Merciful for enabling me to accomplish my research and thesis. May His countless blessings be with us and His last prophet, Muhammad (SAW). I would like to express my sincerest gratitude and appreciation to my supervisor, Associate Professor Dr. Arifah Abdul Kadir, for her extraordinary guidance, commitments, support, and motivation throughout this study. I would also like to thank my co-supervisors, Prof. Dr. Loh Teck Chwen, Prof. Dr. Saleha Abdul Aziz, and Dr. Annas Salleh, for their valuable support, motivation, advice, and encouragement throughout my study. I would like to express appreciation to the Higher Education Commission, Pakistan, and the Bahauddin Zakariya University for the invaluable scholarship. I would also like to thank Geran Putra, Universiti Putra Malaysia, Malaysia, for funding my research grant (Grant Code: G.P./2018/ 9616700).

I wish to express my great thanks to the staff Mr. Johari Ripin (Veterinary Pharmacology Laboratory) and Mr. Kim Wei Chan Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, for their endless assistance and supports throughout this study. My appreciations also go to the staff of the Laboratory of Haematology, Histopathology, Bacteriology, Faculty of Veterinary Medicine, Animal nutrition laboratory, Faculty of Agriculture, Biodiversity unit (UBD), Natural medicine and product research laboratory (Naturmeds), Microbial culture collection unit (UniCC) Institute of Biosciences and Strength of Material Laboratory, Faculty of Engineering, Universiti Putra Malaysia. My appreciation and thanks to all my supportive colleagues in the Faculty of Veterinary Medicine who have been together with me throughout this study period. My heartfelt thanks and love go to my family, especially my wife, son, and daughter, for their endless love and strong mental support throughout my study journey.

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date: 19 May 2022

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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AGPs	Antimicrobial Growth Promoters
PAFs	Phytogenic Feed additives
PBLM	*Piper betle* leaf meal
POLM	*Persicaria odorata* leaf meal
TPC	Total Phenolic Content
TFC	Total Flavonoids Content
TTC	Total Tannins Content
HAL	Halquinol
DMSO	Dimethyl Sulfoxide
GC-MS	Gas Chromatography–Mass Spectrometry
HPLC	High-Performance *Liquid Chromatography*
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
CMC	Carboxymethyl Cellulose
LD_{50}	Lethal Dose Fifty
ICP-MS	Inductively Coupled Plasma Mass Spectrometer
LOD	Limit of Detection
OECD	Organization of Economic Cooperation and Development
BWG	Body Weight Gain
FI	Feed Intake
FCR	Feed Conversion Ration

ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
CFU	Colony Forming Unit
AID	Apparent Ileal Digestibility
TiO_2	Titanium Dioxide
CP	Crude Protein
DM	Dry Matter
EU	European Union
NRC	National Research Council
Mg	Milligram
mmol/L	Milimol Per Liter
g/kg	Gram Per Kilogram
µm	Micrometer
µL	Microliter
a*	Redness
b*	Yellowness
L*	Lightness
TP	Total protein
%	Percentage
˚C	Degree Celsius
SAS	Statistical Analysis System
SEM	Standard Error of Mean

CHAPTER 1

INTRODUCTION

1.1 Introduction

The rapidly increasing human population resulted in an increased demand for animal-source protein. Broiler chickens` meat offers a considerable and affordable potential to fulfil the human needs of animal source dietary protein (Skarp *et al.*, 2016; Mund *et al.*, 2017; Forte *et al.*, 2018). Intensive broiler chicken farming has been carried out to fulfil the continuously increasing demand for poultry meat. In several countries except for Europe, in-feed supplementation of sub-therapeutic doses of antimicrobial growth promoters (AGPs) has been extensively practised to improve feed efficiency and gut health; hence, to achieve the enhanced growth performance of broiler chickens (Abudabos *et al.*, 2016; Alhidary *et al.*, 2017; Mehdi *et al.*, 2018; Proctor A and Phillips GJ, 2019).

However, this indiscriminate use of AGPs in broilers` diets resulted in the emergence of antimicrobial-resistant microorganisms that may be transferring the resistant genes to the microorganisms pertinent to human health (Boovaragamoorthy *et al.*, 2019; Ma *et al.*, 2020).

Antimicrobial resistance is one of the major global concerns (Hedman *et al.*, 2020). Therefore, European Union (EU) and many other countries have banned the in-feed supplementation of AGPs in food-producing animals ((EC) No 1831/2003; Van Boeckel *et al.*, 2015; Tehseen *et al.*, 2016; Tang *et al.*, 2019).

On the other hand, the removal of in-feed AGPs from the poultry diet has exposed the birds to pathogenic organisms and significantly increased the prevalence of infectious diseases (Kumar *et al.*, 2018). Thus, the removal of AGPs from the poultry diet has a negative effect on the production performance of broiler chickens.

In this scenario, it is vital to find potential alternatives that can replace AGPs as feed additives in food-producing animals (Lilleho *et al.*, 2018). The ban against in-feed inclusion of AGPs has increased momentum to find potential alternatives (Al-Abd *et al.*, 2015; Attia *et al.*, 2018). Thus, extensive research has been carried out to find alternatives with similar antimicrobial and growth-promoting potential in broiler chickens (Yang *et al.*, 2015; Rashid *et al.*, 2020). Numerous alternative feed additives like Prebiotics, probiotics, postbiotics, enzymes, organic acid, and phytogenic fed additives (PFAs) or phytobiotics have been studied as a growth promoter in broiler chickens. However, natural substances like herbs, plants, plant extracts, and their essential oils have gotten attention as potential feed additives in poultry production (Gadde *et al.*, 2017; Özbudak, 2019). Earlier studies have suggested that phytobiotics might be the potent and viable natural alternative to AGPs (Murugesan *et al.*, 2015; Yang *et al.*, 2015; Wati *et al.*, 2015; Díaz Carrasco *et al.*, 2018).

Among phytobiotics, herbs are of particular significance because of their secondary bioactive metabolites, such as polyphenols, flavonoids, and phenolic compounds. The herbs and their secondary bioactive compounds are potent antioxidants and antimicrobials that can prevent oxidative stress and reduce the risk of chronic diseases (Pietrzyk *et al.*, 2017; Abbas *et al.*, 2017). Additionally, they are anti-inflammatory, immunomodulatory (Grashorn *et al.*, 2010; Muthusamy *et al.*, 2015), detoxifying, and digestion-stimulating substances (Ganguly *et al.*, 2017). Previous studies showed that herbs positively affect the performance and biological health of broiler chickens (Abudabos *et al.*, 2016; Raza *et al.*, 2016). Moreover, herbs can improve haematological indicators and serum biochemical attributes (Gilani *et al.*, 2018; Odetola *et al.*, 2019; Basit *et al.*,2020b) and have also been reported to regulate kidney and liver functions (Lu *et al.*, 2016; Klaric *et al.*, 2018). Several other beneficial effects of phytobiotics have also been reported in broiler chicken production, including improvement in gut health (Ahsan *et al.*, 2018; Hussein *et al.*, 2020; Basit *et al.*, 2020a) and positive modulation of gut microbiota (Yadav *et al.*, 2019; Basit *et al.*, 2020c). Furthermore, phytobiotics can enhance feed efficiency (Suresh *et al.*, 2018; Tang *et al.*, 2019), thus increasing the growth performance in broilers (Paraskeuas *et al.*, 2017; Mashayekhi *et al.*, 2018; Rahman *et al.*, 2018; Saleh *et al.*, 2018). Additionally, numerous studies have indicated the antibacterial efficacy of phytobiotics against pathogenic bacteria, Like *Clostridium*, *Salmonella*, *Staphylococcus aureus*, and *E. coli* in broiler chickens (Mohiti-Asli *et al.*, 2017; Abudabos *et al.*, 2018; Basit *et al.*, 2020c). On the other hand, one of the primary modes of action of phytobiotics is the balancing of beneficial intestinal microbiota by limiting the pathogenic microorganism (Stevanović *et al.*, 2018; Vase-Khavari *et al.*, 2019).

Natural products like herbs have numerous beneficial and growth-promoting effects in animals and are generally considered safe; however, they may contain some toxic substances (Guldiken *et al.*, 2018; Van *et al.*, 2020). Thus, there are some concerns against the safe use of herbs (Giannenas *et al.*, 2020). The toxicity evaluation of plant-based feed additives can reveal the possible detrimental effects; thus, helping to establish evidence for their safe use (Srinivasa *et al.*, 2018; Williamson *et al.*, 2020). Nevertheless, the data are scarce about the possible detrimental effects of in-feed inclusion of phytobiotics (Karaskova *et al.*, 2015). Hence, it is essential and within scope to investigate the full potential and toxicity evaluation of herbal feed additives, to establish their safe use (Salehi, B *et al.*, 2018; Sharifi-rad *et al.*, 2018).

Among various valuable herbs, one is *Piper betle*. It is an essential herb of the *Piperaceae* family and is widely grown in Southeast Asia and East Africa (Umar *et al.*, 2018). The *P. betle* is a native herb of the central and eastern part of peninsular Malaysia (Pin *et al.*, 2011; Periyanayagam *et al.*, 2012). *Piper betle* is an evergreen climbing shrub producing woody stems 5 - 20 metres long. It is highly abundant and inexpensive, therefore promoting further research and industrialization development, including in the food and pharmaceutical industries. However, its annual yields are estimated at 6 - 10 tonnes per hectare (Pradhan *et al.,* 2013; Nayaka *et al.,* 2021). In Malaysia and Indonesia, it is commonly named as "*daun sirih*." The *P. betle* possesses nutritive and therapeutic properties; thus, widely used in traditional medicinal systems (Farooqui *et al.*, 2016; Biswajit Patra *et al.*, 2016; Taukoorah *et al.*, 2016). This *P. betle* have several biological properties, including antimicrobial (Foo *et al.*, 2015; Chouhan *et al.*, 2017; Singh *et al.*, 2019), antifungal (Basak & Guha, 2017), anti-inflammatory (Biswajit Patra *et al.*, 2016), and antioxidant (Rintu *et al.*, 2015; Kamath & Sabeena, 2018). The reported bioactive compounds of *P. betle* are hydroxychavicol, eugenol, methyl eugenol, and some sterols (Muruganandam *et al.*, 2017). The phenolic compounds of *P. betle* like hydroxychavicol, eugenol, isoeugenol possess potent antioxidant potential (Ali *et al.*, 2018). Previous studies reported the antimicrobial and radical scavenging activities of bioactive compounds (eugenol and isoeugenol) of *P. betle* (Syahidah *et al.*, 2017; Zhang *et al.*, 2017).

Another herb, *Persicaria odorata*/ *Polygonum minus* Huds of family *Polygonaceae*, has been widely studied for its therapeutic use. *Persicaria odorata* is perennial herb up to 0.35 m tall. The foliage retention of *P. odorata* is evergreen. is a culinary herb which is indigenous to Tropical south Asia (Sim *et al.*, 2019). This herb has several common names like Vietnamese cilantro and Vietnamese mint. In Malaysia, Indonesia, Singapore, and Brunei, it is called "*daun laksa*" or "*daun kesum*" (Vikram *et al.*, 2014; Dash & Zakaria 2016). Previous literature has shown that *P. odorata* is a strong antioxidant (Christapher *et al.*, 2016; Abdullah *et al.*, 2017) and antimicrobial (Saad *et al.*, 2014; Abubakar *et al.*, 2015). The secondary bioactive compounds like quercetin, myricetin, and gallic acid are important flavonoids of *P. odorata* (Imelda *et al.*, 2014; Christapher *et al.*, 2017; Pawłowska *et al.*, 2020). These compounds are assumed to be responsible for the antioxidant activity of this herb (Ahmad *et al.*, 2014; Christapher *et al.*, 2015). Moreover, previous studies have shown that *P. odorata* was non-toxic in the murine model (Aprianti *et al.,* 2017; Christapher *et al.*, 2017).

1.2 Problem Statement

The rapidly rising demand for animal-source protein is soaring worldwide. To meet the increasing demand for animals` protein, antibiotics are extensively used in broiler chicken production to achieve maximum growth. This excessive subtherapeutic use of AGPs has resulted in the emergence of antibiotic resistance. Finding novel alternatives to AGPs as growth promoters is much needed. Numerous studies highlighted the positive effects of phytobiotics like herbs as alternative growth promoters in broiler chickens (Khan *et al.*, 2017; Paraskeuas *et al.*, 2017; Mashayekhi *et al.*, 2018; Oso *et al.*, 2019). However, literature is scarce about the safe use of herbal plants and their optimal dosage. Additionally, limited studies reported positive effects of herbs containing tannins in broiler chickens production (Starcevic *et al.*, 2015; Bee *et al.*, 2017). This limitation was due to the belief that tannins might have an anti-nutritional effect when supplemented in broiler chickens` diet (Mansoori *et al.*, 2015). In this study, *P. betle* and *P. odorata* leaf meal were evaluated for the first time as the potential alternative to AGPs in broiler chickens.

1.3 Objectives

This research was carried out to fulfil the following objectives:

1. To assess the qualitative and quantitative phytochemical screening, antimicrobial and antioxidant potential of *Piper betle* and *Persicaria odorata*.

2. To quantify secondary metabolites and assess the acute oral toxicity of *Piper betle* and *Persicaria odorata* leaf extract in broiler chickens to establish their safe use.
3.
4. To determine the effects of graded dose supplementation of *Piper betle* and *Persicaria odorata* leaf meal in broiler chicken feed on growth performance, apparent ileal digestibility, gut morphology, haematological indices, serum biochemical profile, and liver histomorphological changes.

5. To investigate the comparative efficacy of phytobiotics (*Piper betle* and *Persicaria odorata* leaf meal) with halquinol and tetracycline on growth performance, gut morphology, ileal digestibility, meat quality, tibia bone morphometric characteristics, and caecal microbiota composition in broiler chickens.

1.4 Hypothesis

It is hypothesised that:

1. The *Piper betle* and *Persicaria odorata* leaf extracts would show antimicrobial and antioxidant potential with successful quantification of secondary bioactive compounds like total phenolic content, total flavonoids, and total tannins.

2. Secondary bioactive compounds eugenol and quercetin would be successfully quantified from the *Piper betle* and *Persicaria odorata* methanolic leaf extract; also, these extracts would show no acute oral toxicity in broiler chickens.

3. Graded dose supplementation of *Piper betle* and *Persicaria odorata* leaf meal would show positive effects on apparent ileal digestibility, improve gut morphology, have no deleterious impact on haematological indices, serum biochemical profile, and liver morphology, thus can enhance growth performance in broiler chickens.

4. Supplementation of *Piper betle* and *Persicaria odorata* leaf meal would show comparable or better performance to halquinol and AGPs as alternative growth promoters in sustainable broiler chickens production.

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