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QUANTITATIVE PHYTOCHEMICAL SCREENING AND ACUTE ORAL TOXICITY STUDY OF PIPER BETLE AND PERSICARIA ODORATA LEAF EXTRACT IN BROILER CHICKENS

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Abstract

Medicinal plants like herbs are used extensively as an alternative poultry feed additive, replacing antimicrobial drugs. However, these herbs and their bioactive compounds may contain toxic substances that may be harmful. Thus, the present study was conducted to estimate the secondary phytochemical metabolites and acute toxicity of methanolic leaf extract of *Piper betle* (Pb) and *Persicaria odorata* (Po) in broiler chickens. A total of 35 broiler chicks were used in this study. The birds were divided into 7 groups randomly on the 21st day of their age. A single oral 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 body weight, was orally gavaged in treated chickens. At the same time, the control group received 0.5% carboxymethyl cellulose (CMC) as a placebo. Quantitative phytochemical screening showed positive quantification of eugenol and quercetin from *Pb* and *Po* methanolic leaf extract. On the other hand, the limited dose acute toxicity study did not show any toxicity signs, even at the dose

rate of 2000 mg/kg. Furthermore, no deleterious effects were observed on haematological and serum biochemical indices in extract-treated chickens. The histopathological examination showed normal histo-morphological characteristics of the tissues of selected organs. Additionally, no mortality was recorded in the entire study period. Keeping in view the present findings, the LD_{50} value of the tested extracts was greater than 2000 mg/ kg.

Keywords: broiler chickens; herbs; limit dose toxicity; *Piper betle*; *Persicaria odorata*

Introduction:

Antimicrobial resistance is an important global concern [1]. The persistent infeed sub-therapeutic supplementation of antimicrobial growth promoters (AGPs) possibly resulted in the multidrug-resistant microbial population [2, 3]. Natural feed additives are extensively screened in food-producing animals to replace conventional feed additives like subtherapeutic AGPs [3]. Phytobiotics, like herbs, have gained attention because of their relative safety, which has triggered extensive research in botanical feed additives [4, 5].

Natural products like herbs are generally considered safe, but on the other hand, these medicinal plants or herbs may have some toxic secondary compounds [6, 7]. Thus, using phytobiotics as animal feed additives might not be completely safe without toxicity evaluation [8, 9]. Hence, the toxicity screening of natural products helps to devise their safe use [10, 11]. Moreover, limited scientific information is available about the potentially harmful effects of the dietary supplementation of phytobiotics [12, 13]. Hence, it is crucial to investigate the toxicity screening of phytobiotics to ascertain their safe use [14].

Piper betel is a herb in Malaysia of the *Piperaceae* family. It possesses nutritional and medicinal values [15]. Additionally, *P. betel* leaves possess significant antimicrobial, antifungal [16, 17], and antioxidant properties [17]. The bioactive compounds of *P. betel* include terpenes and phenols like chavibetol, hydroxychavicol, eugenol, methyl eugenol, and some sterols [18]. Among bioactive compounds, the most significant are eugenol and hydroxychavicol; these secondary metabolites have been established as potent antimicrobials [17, 19].

Persicaria odorata, synonymously called *Polygonum minus* Huds, is a Polygonaceae herb. It is used traditionally in Southeast Asian cuisine [20]. This herb is commonly named "*daun laksa*" or "*daun kesum*" [21]. *P. odorata* is a strong antioxidant herb [22]. The *P. odorata* is enriched with flavonoids, including quercetin, myricetin, and gallic acid [23]. The bioactive compounds quercetin and myricetin are assumed to be responsible for antioxidant activity [17, 24, 25], which can also inhibit lipid peroxidation [26]. Previous studies designated that *P. odorata* is nontoxic in the murine model [27, 28].

The current study intended to evaluate the secondary phytochemical metabolites and acute toxicity of methanolic leaf extract of *Piper betle* (Pb) and *Persicaria odorata* (Po) in broiler chickens. This study is the first of its kind to assess the limit dose acute toxicity of selected herb leaves extracts in chickens.

Material and methods:

Collection and identification of plant samples:

The collection and identification of fresh samples of *P, odorata* and *P. betle* were in line with (17). Briefly, fresh leaves were harvested from the Herbarium Garden at Universiti Putra Malaysia (UPM) and authenticated from the Biodiversity Unit, Institute of Biosciences, UPM.

Preparation of methanol extract:

The methanolic extract of *Pb* and *Po* was prepared as described previously by [17]. Briefly, the collected leaves of *Pb* and *Po* were washed with purified water. The leaves were air-dried at room

temperature and oven-dried until they got a constant weight at 50 °C. Finally, these leaves were grounded to get the fine powder. The botanical powders of *Po* and *Pb* (10g) were extracted with 150 mL of methanol in a shaking incubator (150 rmp, 25 °C) for 24h. After that, the mixtures were filtered through Whatman filter paper No. 1. Later, the residues were re-extracted according to the same procedure. The filtrates were pooled and dried under reduced pressure at 40 °C using a rotary evaporator (Heildoph HB 4000, USA), followed by oven drying at 40 °C overnight. Finally, the extraction yield was determined gravimetrically [29], and the *Pb* and *Po* leave extracts were stored at -20 °C.

$$Yield\ (\%) = \frac{Wii - Wi}{Ws} \times 100$$

Where W*ii* is the weight of the extract and container, W*i* is the weight of the empty container, and Ws is the weight of the initial dried sample.

Sample Preparation for Oral Gavage:

The methanolic leaf extracts of *Pb* and *Po* were suspended in the carboxymethylcellulose (CMC, 5 %) and then sonicated to constitute the desired dose. The extracts were orally gavaged to the experimental birds.

Gas chromatography-mass Spectrophotometry methanolic leaf extract of *P. odorata and P. betle*:

The metabolite/ compounds in the methanolic leaves extract of *P. betle* and *P. odorata* were analysed by using the GC-MS (GCMS-QP2010 Ultra, Shimadzu, Kyoto, Japan) according to the method previously described by [30] and [31] with minor modifications**.** Chromatography conditions included a capillary column (model Rxi-5ms; 30.0m Length x 0.25mm ID x 0.25µm Thickness, Shimadzu, Kyoto, Japan). Furthermore, the sample volume was 0.5 mL, while the carrier gas (helium) was used at a flow rate of 0.80 mL/min; the column oven temperature was 50 °C. The mass spectra were obtained by electron ionisation at 85 eV, and the detection range was 40-700 m/z. However, compounds were identified using the database of NIST (edition 11s) and WILEY (edition 229) mass spectral libraries.

Heavy Metal Analysis:

The analytical measurement of cadmium (Cd), lead (Pb), and arsenic (As) in the methanolic leaves extracts of *P. betle* and *P. odorata* were evaluated using an inductively coupled plasma mass spectrometer ICP-MS (Agilent 7700x, Barcelona, Spain) in line with the method described by [32].

Quantification of Eugenol and Quercetin:

The eugenol and quercetin were successfully quantified from *Pb* and *Po* methanolic leaves extracts, respectively. Quantification of eugenol and quercetin was achieved using high-performance liquid chromatography (HPLC) according to the methods designated and validated by [19] for eugenol and [33] for quercetin.

Chromatographic Conditions for Eugenol and Quercetin Quantification:

Quantification was performed using an HPLC isocratic system (Agilent 1260, DE, USA), equipped with a degasser (G1379A) and autosampler (G1311A). The Luna C18 (250 × 4.6 mm, 5 µm) column was used for chromatographic separation. The data was collected and analysed using a chem station (Agilent Technologies, Open lab Revision A.02.19 (14.5)). The eugenol was eluted using a mobile phase made up of acetonitrile and 1% acetic acid (40:60 v/v). Eugenol was detected at 280 nm wavelength, while the flow rate was 1 ml/min. The retention time was 7.16 min. On the other hand, quercetin was eluted using a mobile phase made up of methanol and 0.1 % orthophosphoric acid (65:35 v/v). Quercetin detection was done at 370 nm wavelength, while the flow rate was 1 ml/min. The retention time was 8.10 min.

Eugenol and quercetin were identified by comparing the retention time with the authentic standard. The quantification of compounds was attained by directly comparing the peak area ratios of the samples to the authentic standard compound used as standards.

Acute Toxicity Study:
The acute toxicity test was carried out according to the guiding principle of the Organization of Economic Cooperation and Development (OECD) for testing of chemicals (OECD, 223) [34]. The experiment was carried out in line with the approved procedures of the Institutional Animal Care and Use Committee, UPM (UPM/IACUC/AUP-R033/2018).

Experimental Design:
Thirty-five one-day-old broiler chicks (Cobb 500) were received from a local hatchery. Upon arrival, the birds were individually wing-banned. On day 21st of their age, the body weight of the birds was measured (810-820 g); later, the birds were randomly distributed into 7 groups. Each group consisted of five birds. The birds were raised in battery cages under the same environmental and managemental conditions. The vaccination and brooding of the chicks were conducted in line with [35]. The birds were provided with feed and water *ad libitum* while the lighting was continuous.
The birds were administered single oral doses of methanolic leaves extracts of *P. betle* and *P. odorata* into the crop by gavage tube. Group-1 received 0.5% CMC as placebo and served as the control; groups 2-4 (Pb 500; Pb 1000 and Pb 2000) received methanolic leaf extract of *P. betle*, while groups 5-7 (Po 500; Po 1000 and Po 2000) received methanolic leaf extract of *P. odorata* at the rate of 500 mg/kg, 1000 mg/kg and 2000 mg/ kg of body weight respectively. The chicks were offered a basal diet throughout the experiment (starter diet 1-21 days and finisher diet 22-35 days) that meets or exceeds National Research Council recommendations NRC (1994) [36].

Observational and Behavioral Study:
The birds were observed for food consumption and water intake. The behavioural and physical abnormalities, including inappetence, ataxia, dropping of wings, diarrhoea, and convulsions, were observed after oral gavage within the first 15 minutes, 30 minutes, 2 h, 4 h, 6 h, up to 24 h, and once daily till 14 days [37,38]. The body weight gain (BWG) and feed intake (FI) were measured weekly to calculate the feed conversion ratio (FCR). The events of mortality were recorded throughout the experimental period.

Haematological and Biochemical Analysis:
On day 35, blood samples were collected from the brachial (wing) vein of each bird for haematological analysis in the EDTA (K_3) tubes (BD Vacutainer®, NJ, USA). While for the serum biochemical analysis, the samples were obtained in plain tubes (BD Vacutainer®, NJ, USA), and the serum was harvested, subjecting the samples to centrifuge at 3000 × g for 15 min and stored at -20 °C. The haematological analysis was performed using an "ABX Diagnostics" haematology analyser (ABC Vet®, Montpellier, France). The blood haematology profile included haemoglobin (Hb), white blood cell (WBCs) & red blood cell (RBCs) counts, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentrations (MCHC), and packed cell volume (PCV). The serum biochemistry profile was performed using a chemistry auto analyser (Bio Lis 24i, Tokyo, Japan). The biochemical indices included alkaline phosphatase (ALP), alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), glucose, total protein (TP), albumin, globulin, cholesterol, triacylglycerols, creatinine, urea, and serum electrolytes; potassium (K), chloride (Cl), and sodium (Na).

Organ Weights and Histopathology Evaluation:
At the end of the experiment, all birds were slaughtered in accordance with the guidelines of the (Malaysian Standard (MS) 1500: 2009) [39]. Weights of internal organs, including the liver, heart,

lungs, kidney, spleen, pancreas, and gizzard, were obtained and expressed as a percentage (%) of the live body weights of the birds. Furthermore, the tissue samples of harvested organs were preserved in 10 % buffered formalin. The tissue sections were processed for histopathological evaluation as described by [40]. Each section of the selected organ is processed through a series of dehydration cycles in an automated tissue processor (Leica ASP 3000, Wetzlar, Germany). Later, these tissue sections were embedded using a paraffin embedding station (EG1150 II, Leica, Wetzlar, Germany). The tissue sections were obtained up to 4-5µm with a sectioning rotary microtome (RM2045, Leica Wetzlar, Germany). The staining was performed using hematoxylin and eosin staining. The histomorphological examination of the tissues was performed using a light microscope (Leica DM LB2, Wetzlar, Germany).

Statistical Analysis:

The obtained data were analysed by one-way ANOVA using general linear models (GLM) procedures of Statistical Analysis System software (SAS) version 9.4 [41]. Group differences were then elucidated and compared by Tukey's posthoc test. Statistically significant differences were expressed with a significant ($p < 0.05$) level.

Results:

Percentage Yield of Extracts:

The obtained yields from 10 grams of dried leaves of *P. betle* and *P. odorata* were 15.25 and 15.75%, respectively.

GC-MS Analysis:

GC-MS analyses of *P. betle* and *P. odorata* methanolic leaf extracts indicated the presence of metabolites shown in (Table 1 & Table 2). The GC-MS analyses of methanolic leaf extract of *P. betle* designated the predominant presence of phenolic monoterpenes hydroxyphenyl propene, like eugenol, m-eugenol, and Allylbenzene-1,2-diol-Hydroxychavicol. On the other hand, the GC-MS analyses of the

methanolic leaf extract of *P. odorata* designated the predominant occurrence of terpenes and aliphatic compounds like nonanal, hexadecanal, and β-Caryophyllene.

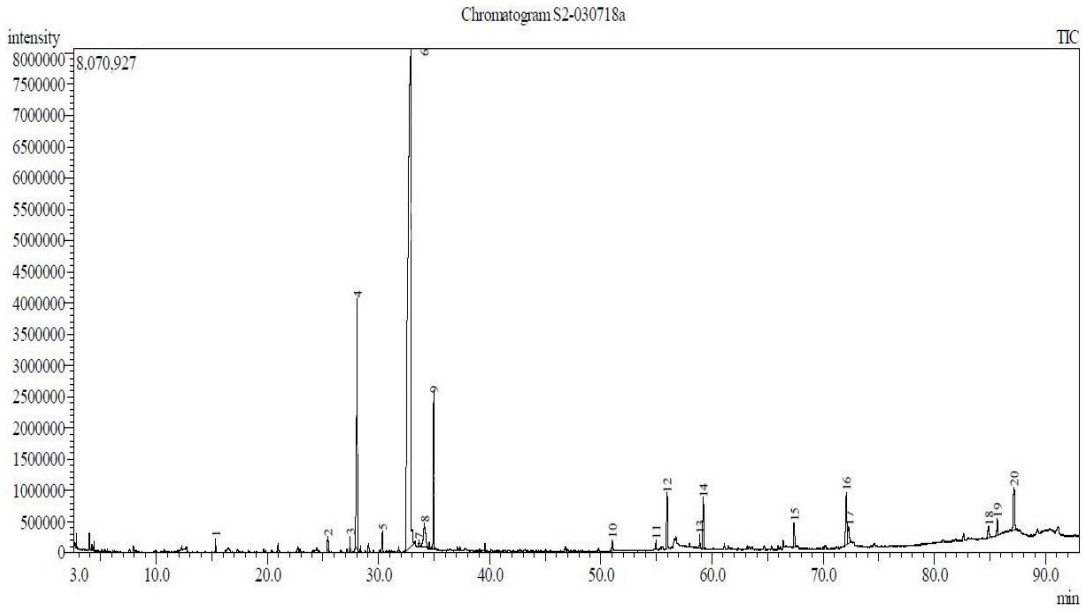

Fig. 1 GC-MS Chromatogram of methanolic leaf extract of *P. betle*

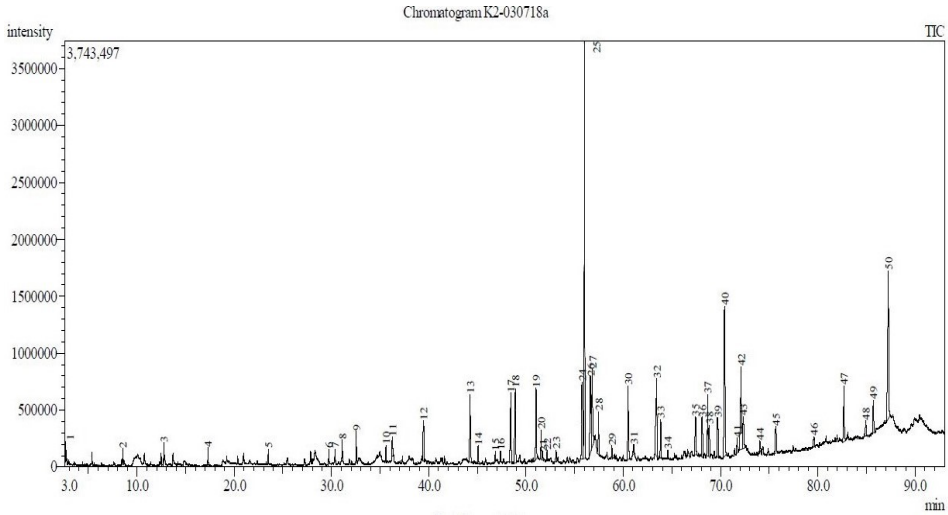

Fig. 2 GC-MS Chromatogram of methanolic leaf extract of *P. odorata*

Table 1: GC-MS analyses of phytocomponents in the methanolic leaf extract of *P. betle*

Compound/ Metabolite *	**Formula**	**Content (%)**
4-Allylbenzene-1,2-diol-Hydroxychavicol	$C_9H_{10}O_2$	2.42
Phenol, 2-methoxy-3-(2-propenyl)-Eugenol	$C_{10}H_{12}O_2$	10.24
Chavibetol (2-Methoxy-5-(2-propenyl) phenol); m- Eugenol	$C_{10}H_{12}O_2$	5.75
2,5-Dimethoxybenzoic acid	$C_9H_{10}O_4$	16.11
trans-Phytol, (2E,7R,11R)-Phytol	$C_{20}H_{40}O$	1.89
Piperin; 1-Piperoylpiperidine	$C_{17}H_{19}NO_3$	1.04
9,12-Octadecadienoic acid - Linolein	$C_{21}H_{38}O_4$	2.76
Stigmasta-5,24(28)-dien-3-ol	$C_{29}H_{48}O$	2.37
β-sitosterol,	$C_{29}H_{50}O$	1.03

*Compounds identified by GC-MS software, and the naming was according to the NIST (edition; 11s) and WILEY (edition; 229) mass spectral libraries.

Table 2: GC-MS analyses of phytocomponents in the methanolic leaf extract of *P. odorata*

Compound/ Metabolite *	**Formula**	**Content (%)**
β-Caryophyllene	$C_{15}H_{24}$	3.32
trans-Sesquisabinene hydrate-Nerolidol	$C_{15}H_{26}O$	0.92
Nonanal	$C_9H_{18}O$	6.76
Phytol	$C_{20}H_{40}O$	15.29
Decanal	$C_{10}H_{20}O$	4.73
n-Dodecanol	$C_{12}H_{26}O$	0.51
1-Decanol	$C_{10}H_{22}O$	1.59
Tridecane	$C_{13}H_{28}$	1.10
trans-α-Bergamotene	$C_{15}H_{24}$	1.50
Undecanoic acid	$C_{11}H_{22}O_2$	0.56
Drim-7-en-11-ol- Drimenol	$C_{15}H_{26}O$	2.90
Hexadecanal	C16H32O	4.13
Dodecanoic acid, dodecyl ester	$C_{24}H_{48}O_2$	2.29
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	3.15
beta-Tocopherol	$C_{29}H_{50}O_2$	2.24

*Compounds identified by GC-MS software, and the naming was according to the NIST (edition; 11s) and WILEY (edition; 229) mass spectral libraries

Quantification of Eugenol and Quercetin HPLC Analysis:

The HPLC technique was employed to quantify eugenol from *P. betle* and quercetin from *P. odorata*; the analyses confirmed the presence of eugenol and quercetin in *P. betle* and *P. odorata*, respectively. The chromatograms of reference standards and phyto-compounds are shown in Fig: 3 (a-b) & fig. 4 (a-b). The calculated amounts of bioactive compounds eugenol and quercetin were 56.58 and 22.68 mg/g, respectively. In addition, the contents of the standard compound in 10 g dried leaves of *P. betle* and *P. odorata* were 86.28 mg of eugenol and 35.72 mg of quercetin, respectively (Table 3).

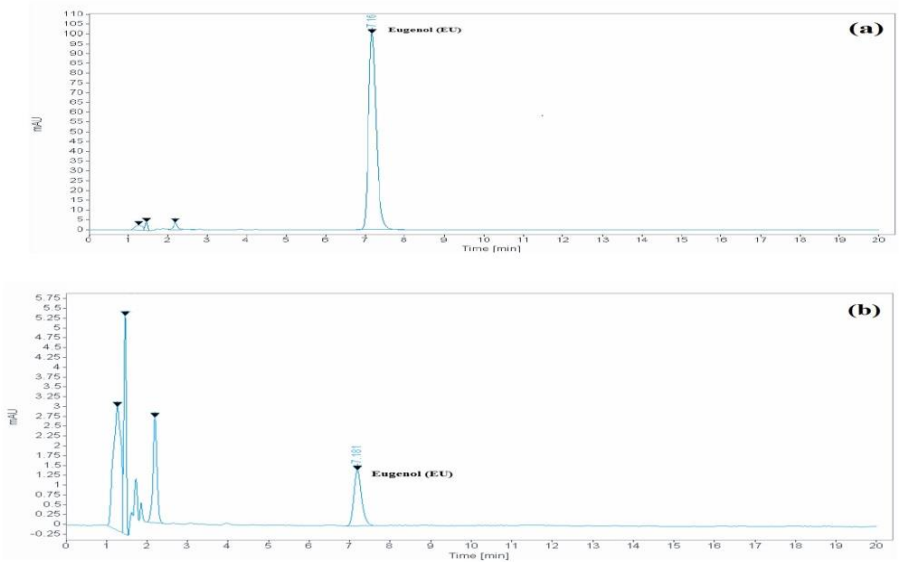

Fig. 3 (a-b): (a) Reference chromatogram of eugenol (standard) (b) Chromatographic analyses of methanolic leaf extract of *P. betle*

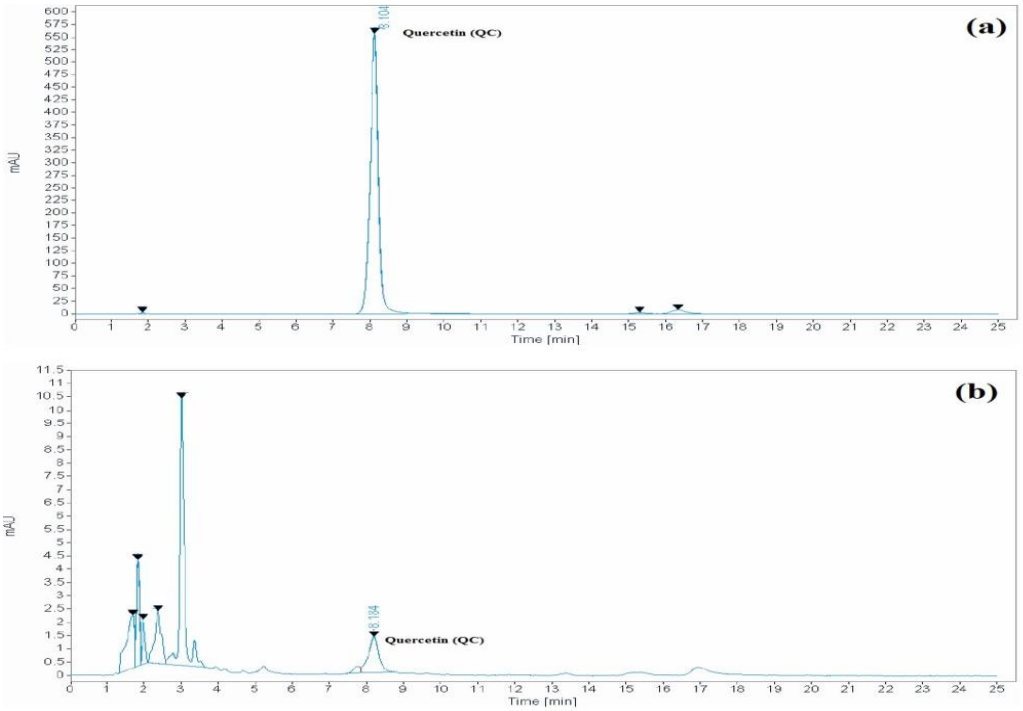

Fig. 4 (a-b): (a) Reference chromatogram of quercetin (standard) (b) Chromatographic analyses of methanolic leaf extract of *P. odorata*

Table 3: Quantification of eugenol in methanolic leaf extract of *P. betle* and Quercetin in *P. odorata* using chromatographic analyses

Sample	Compound	Content of standard compound (mg/g)	Content of standard compound (mg) in 10 gm of dried leaves
P. betle (Pb)	Eugenol	56.58 ±0.17	86.28 ±0.18
P. odorata (Po)	Quercetin	22.68 ±0.16	35.72 ±0.19

(n=3)

Metal Analysis:

The estimation of cadmium (Cd), lead (Pb), and arsenic (As) were performed using ICP-MS. The analytical quantifications of the heavy metals were found below the detection limit (Table 4). The limit of detection (LOD) for Cd, Pb, and AS for the current method was 0.046, 0.06, and 0.02 ppb, respectively.

Table 4: Metal contents (ug/g) of methanol extract of *P. betle* and *P. odorata* leaves

Heavy Metal	The concentration of heavy metals	
	P. betle	*P. odorata*
Lead	ND	ND
Cadmium	ND	ND
Arsenic	ND	ND

Not detected (ND)

Acute Toxicity:

Observational Study:

The experimental broiler chickens did not show any change in behaviour after single oral doses of administration. However, the birds showed inappetence during the first 8 h, which was resolved later, and all the birds started consuming feed and water in a regular pattern. The birds showed no toxicity signs; no mortality was recorded during the acute toxicity study period (Table 5).

Table 5: Effect of single limit toxicity dosage of methanolic leaf extract of *P. betle* and *P. odorata* (500, 1000, and 2000 mg/ kg) in broiler chickens

Sign	**Control**	***P. betle***			***P. odorata***		
		Pb500	Pb1000	Pb2000	Po500	Po1000	Po2000
Inappetence (24 h)	P	P	P	P	P	P	P
Ataxia	A	A	A	A	A	A	A
Dropping of wings	A	A	A	A	A	A	A
Diarrhea	A	A	A	A	A	A	A
Convulsions	A	A	A	A	A	A	A
Mortality after (24 h)	0	0	0	0	0	0	0
Mortality till 14 days	0	0	0	0	0	0	0

P= present, A= absent, Number of birds died= 0,1,2…

C (Control); Pb500 (*P. betle* extract 500mg/kg); Pb1000(*P. betle* extract 1000mg/kg); Pb2000(*P. betle* extract 2000mg/kg); Po500 (*P. odorata* extract 500mg/kg); Po1000 (*P. odorata* extract 1000mg/kg); Po 2000 (*P. odorata* extract 2000mg/kg).

Growth Performance:
The bodyweight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) of experimental broiler chickens showed no significant difference on day 7 and day 14 after single oral dose administration (Table 6).

Table 6: Growth measures after single limit toxicity dosage of methanolic leaf extract of *P. betle* and *P. odorata* (500, 1000, and 2000 mg/ kg) in broiler chickens

Parameter	C	*Piper betle*			*Persicaria odorata*			SEM
		Pb500	Pb1000	Pb2000	Po500	Po1000	Po2000	
1-7 days								
BWG (gm)	476.21	483.95	482.35	497.35	482.27	483.69	491.09	3.40
FI (gm)	959.60	958.20	958.64	956.44	959.66	960.46	959.46	1.94
FCR	2.02	1.98	1.99	1.92	1.99	1.99	1.96	0.14
7-14 days								
BWG (gm)	496.18	507.25	518.25	518.85	510.70	514.30	515.30	3.33
FI (gm)	967.01	966.04	967.64	966.44	970.24	968.04	967.16	1.72
FCR	1.95	1.91	1.87	1.86	1.90	1.88	1.88	0.11
1-14 days								
BWG (gm)	972.39	991.19	1000.59	1016.19	992.97	997.99	1006.39	5.25
FI (gm)	1926.62	1924.24	1926.28	1922.88	1929.91	1928.51	1926.63	2.76
FCR	1.98	1.94	1.93	1.89	1.94	1.93	1.92	0.01

BWG: Body weight gain, FI: Feed intake, FCR: Feed conversion ratio SEM= Standard error mean
C (Control); Pb500 (*P. betle* extract 500mg/kg); Pb1000(*P. betle* extract 1000mg/kg); Pb2000(*P. betle* extract 2000mg/kg); Po500 (*P. odorata* extract 500mg/kg); Po1000 (*P. odorata* extract 1000mg/kg); Po 2000 (*P. odorata* extract 2000mg/kg)

Haematology:
Data in Table 7 illustrates the impact of single doses administration of methanolic leaf extract of *P. betle* and *P. odorata* on haematological indicators of blood from broiler chickens. Hematological indices, namely RBC count, PCV, Hb, MCV, MCH, MCHC, and WBC count were not influenced by various concentrations of single-dose administration of methanolic leaf extract of *P. betle* and *P. odorata*.

Serum Biochemical Analysis:
The effect of single doses administration of methanolic leaf extract of *P. betle* and *P. odorata* on serum biochemistry parameters of broiler chickens are shown in (Table 8). Experimental birds did not show any significant difference in serum biochemical indices. However, the activity of AST was reduced ($p < 0.05$) in the extract-treated birds at a dose rate of 2000 mg/kg of methanolic leaf extract of *P. betle* and *P. odorata* relative to the birds of the control group. On the other hand, the serum concentration of urea decreased ($p < 0.05$) in extract-treated groups of *P. betle* (1000 and 2000mg/kg)

and *P. odorata* (2000 mg/kg) compared to the control group. While the serum concentration of creatinine decreased ($p < 0.05$) in extract-treated group *P. betle* (2000 mg/kg) in comparison with the control group.

Table 7: Hematological indices of blood counts after single limit toxicity dosage of methanolic leaf extract of *P. betle* and *P. odorata* (500, 1000, and 2000 mg/ kg) in broiler chickens

Parameters	C	*P. betle*			*P. odorata*			SEM
		Pb500	Pb1000	Pb2000	Po500	Po1000	Po2000	
RBCs ($mm^3 \times 10^6$)	2.20	2.38	2.38	2.41	2.36	2.37	2.40	0.04
PCV (%)	29.89	30.01	30.02	30.36	29.42	29.44	29.92	0.24
Hb (g/dL)	8.89	9.28	9.24	9.35	9.04	9.11	9.18	0.13
MCV (fL)	137.05	126.51	126.16	126.40	125.13	125.20	125.76	1.48
MCH (pg)	40.77	38.95	38.81	38.77	38.27	38.67	38.43	0.37
MCHC (%)	29.77	30.93	30.79	30.74	30.68	30.94	30.64	0.33
WBCs ($mm^3 \times 10^6$)	20.52	20.74	20.89	20.96	20.23	20.30	20.37	0.14

C (Control); Pb500 (*P. betle* extract 500mg/kg); Pb1000(*P. betle* extract 1000mg/kg); Pb2000(*P. betle* extract 2000mg/kg); Po500 (*P. odorata* extract 500mg/kg); Po1000 (*P. odorata* extract 1000mg/kg); Po 2000 (*P. odorata* extract 2000mg/kg)

Table 8: Serum biochemical analysis after single limit toxicity dosage of methanolic leaf extract of *P. betle* and *P. odorata* (500, 1000, and 2000 mg/ kg) in broiler chickens

Parameters	C	***P. betle***			***P. odorata***			SEM
		Pb500	Pb1000	Pb2000	Po500	Po1000	Po2000	
ALP (U/L)	1590.60	1591.20	1589.80	1590.40	1578.17	1574.65	1577.00	7.16
AST (U/L)	222.00 [a]	205.27 [ab]	204.80 [ab]	202.60 [b]	204.82 [ab]	205.34 [ab]	202.62 [b]	1.75
ALT (U/L)	8.11	7.05	7.02	6.96	7.43	7.58	7.30	0.13
Total protein (g/L)	24.41	24.92	25.54	25.57	24.88	25.24	25.50	0.28
Albumin (g/L)	12.01	11.97	12.37	12.52	12.86	13.03	12.42	0.18
Globulin (g/L)	12.39	12.95	13.17	13.05	12.01	12.21	13.08	0.31
Glucose (mmol/L)	13.76	14.27	14.26	13.80	14.65	14.51	14.00	0.30
Cholesterol (mmol/L)	3.02	2.92	2.91	2.90	2.97	2.96	2.95	0.03
Triglycerides (mmol/L)	1.01	0.94	0.93	0.93	1.02	0.97	0.96	0.02
Na (mmol/L)	132.80	134.20	133.58	133.60	135.01	135.86	134.90	1.17
K (mmol/L)	4.40	4.29	4.19	4.20	4.27	4.26	4.16	0.11
Cl (mmol/L)	114.40	111.06	111.27	110.59	108.32	109.54	109.80	1.24
Urea (mmol/L)	0.75 [a]	0.62 [ab]	0.61 [b]	0.60 [b]	0.62 [ab]	0.63 [ab]	0.61 [b]	0.01
Creatinine (µmol/L)	34.40 [a]	28.65 [ab]	27.96 [ab]	24.40 [b]	29.29 [ab]	29.02 [ab]	28.44 [ab]	0.77

[a-b] indicates that values in the same row with different superscripts are significantly different ($p < 0.05$).
C (Control); Pb500 (*P. betle* extract 500mg/kg); Pb1000(*P. betle* extract 1000mg/kg) ; Pb2000(*P. betle* extract 2000mg/kg);
Po500 (*P. odorata* extract 500mg/kg); Po1000 (*P. odorata* extract 1000mg/kg) ; Po 2000 (*P. odorata* extract 2000mg/kg)

Organ Weights:
The relative internal organ weights of broilers administered a single oral dose of methanolic leaf extract of *P. betle* and *P. odorata* showed no difference ($p > 0.05$) compared to the internal organ weights of the control group. (Table 9)

Table 9: Relative internal organ weights of broiler chickens after single limit toxicity dosage of methanolic leaf extract of *P. betle* and *P. odorata* (500, 1000, and 2000 mg/ kg)

		P. betle			***P. odorata***			
Organs	C	Pb500	Pb1000	Pb2000	Po500	Po1000	Po2000	**SEM**
Heart	0.590	0.593	0.591	0.586	0.588	0.577	0.576	0.02
Lungs	0.660	0.671	0.661	0.672	0.663	0.670	0.673	0.01
Liver	2.398	2.349	2.358	2.335	2.354	2.369	2.348	0.08
Spleen	0.149	0.164	0.173	0.176	0.164	0.166	0.166	0.00
Kidney	0.65	0.624	0.628	0.617	0.628	0.624	0.622	0.02
Gizzard	1.993	2.022	2.051	2.034	2.006	2.033	2.034	0.05

C (Control); Pb500 (*P. betle* extract 500mg/kg); Pb1000(*P. betle* extract 1000mg/kg); Pb2000(*P. betle* extract 2000mg/kg); Po500 (*P. odorata* extract 500mg/kg); Po1000 (*P. odorata* extract 1000mg/kg); Po 2000 (*P. odorata* extract 2000mg/kg)

Histopathology:
The tissues of selected organs in all experimental birds showed normal architecture across the experimental groups (figures 5-10 (a-c)).

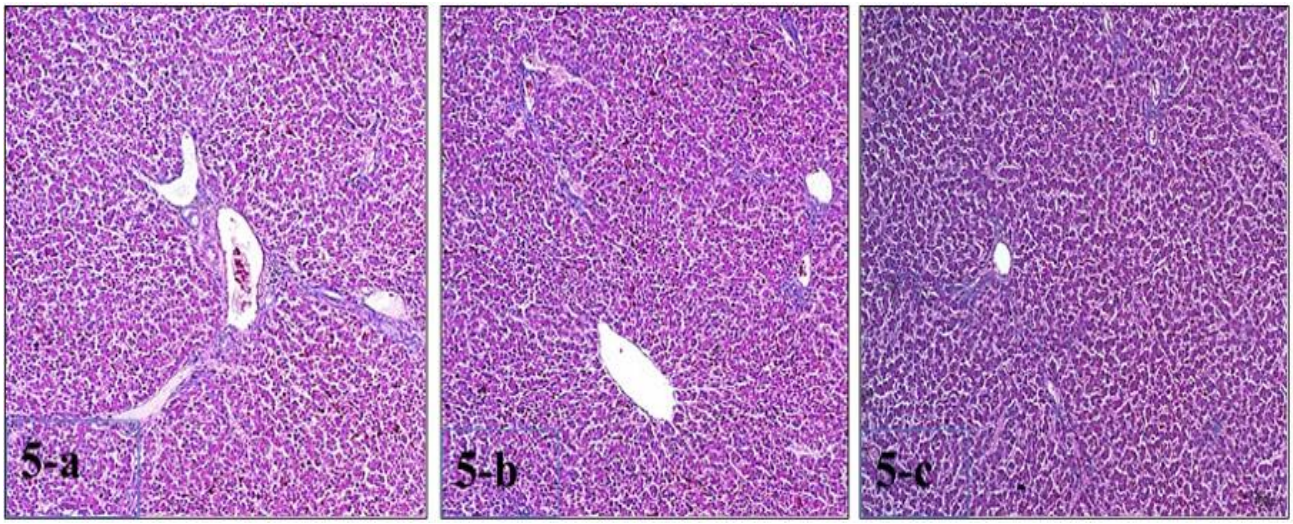

Fig. 5 (a-c): Photomicrograph of liver All the tissues showed normal architecture liver parenchyma.

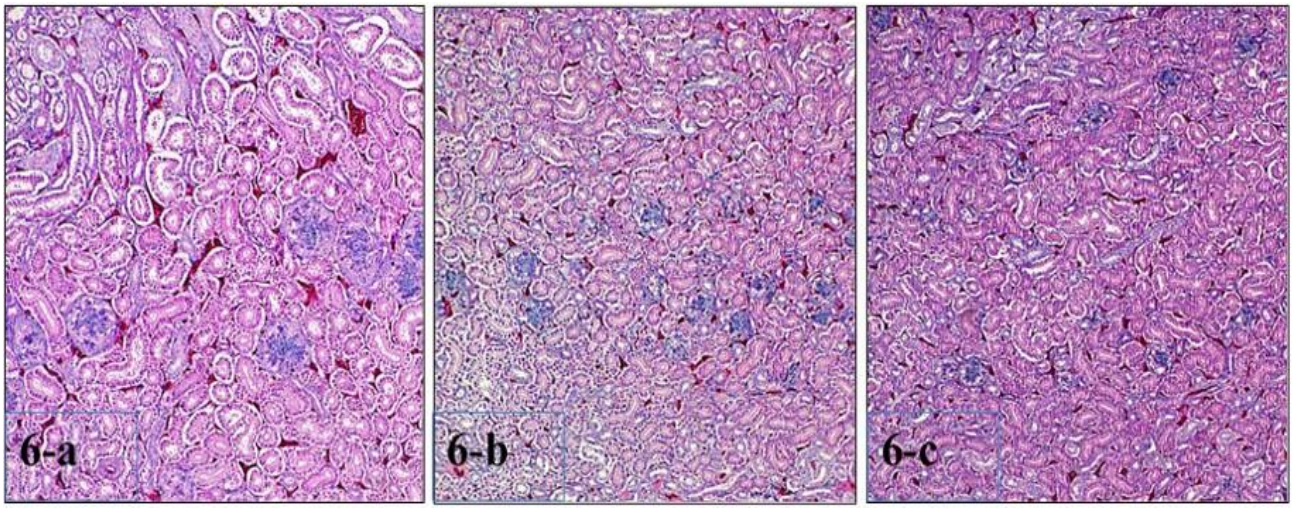

Fig. 6 (a-c): Photomicrograph of Kidney All the tissues showed normal architecture of the renal filtering units (i.e., glomerulus, proximal and distal tubules).

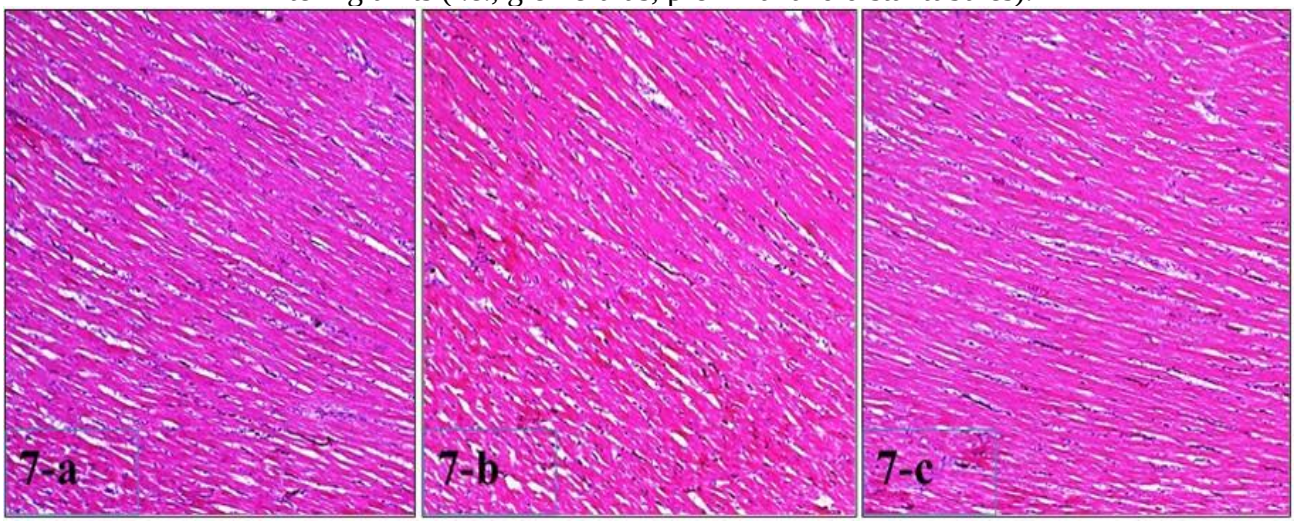

Fig. 7 (a-c): Photomicrograph of heart All the tissues showed normal architecture of myocardial tissue.

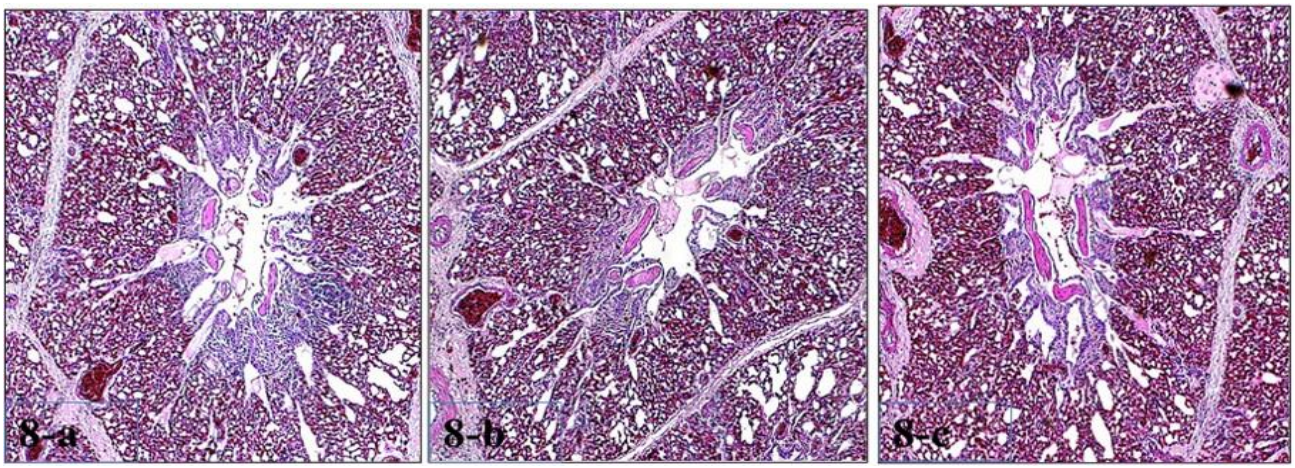

Fig. 8 (a-c): Photomicrograph of lungs All the tissues showed normal architecture of the lung units (parabronchus and surrounding tissues) are normal.

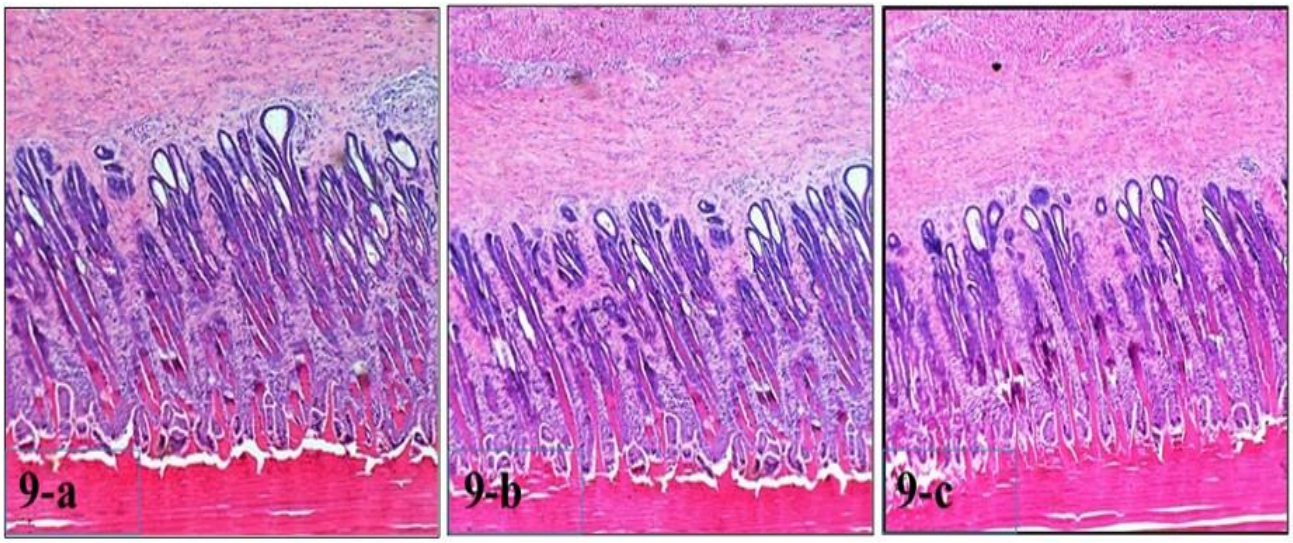

Fig. 9 (a-c): Photomicrograph of gizzard All the tissues showed normal architecture of gizzard tissue (pellicle, secretory lining, and tunica muscularis).

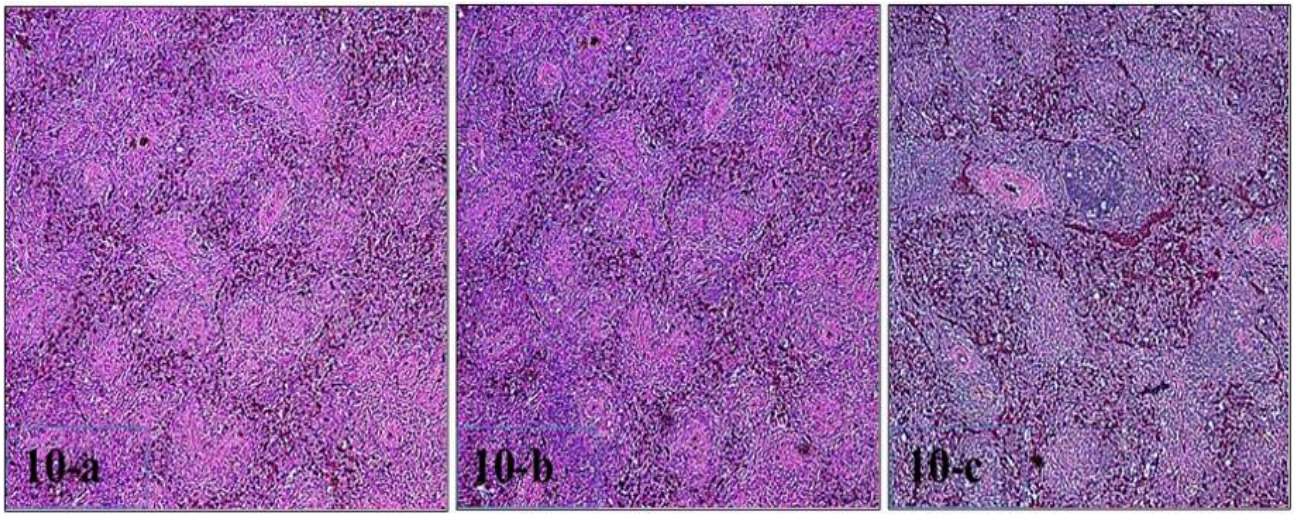

Fig. 10 (a-c): Photomicrograph of spleen All the splenic tissues showed normal architecture of the red and white pulp.

** **Fig. 5-10** (a) control received 5% CMC as Placebo (b) methanolic leaf extract of *P. betle* at the dose rate of (2000mg/kg b.w.) (c) methanolic leaf extract of *P. odorata* at the dose rate of (2000mg/kg b.w.). (H&E: Hematoxylin and Eosin; 100X)

Discussion:

Quantitative Phytochemical Analysis of *P. betle* and *P. odorata*:

Natural products like medicinal plants, herbs, and their bioactive compounds are used extensively as therapeutic alternatives [28;42]. Generally, natural products are considered safe, but scarce literature is available about their possible toxicity and safe use. The *P. betle* and *P. odorata* showed various pharmacological properties reported by [17; 18; 24]. The current study evaluated the quantitative analysis of selected herbs and estimated their acute toxicity in the target species, broiler chickens.

In the current study, the GC-MS analysis of methanolic leaf extract of *P. betle* showed the predominant presence of phenolic monoterpene hydroxyphenyl propene like eugenol, m-eugenol, and Allylbenzene-1,2-diol-Hydroxychavicol. Current study results designated the predominant presence of phenolic compounds like eugenol in the methanolic leaf extract of *P. betle*. Numerous studies highlight that phenolic compounds like eugenol and hydroxychavicol are antimicrobial, antioxidant, and anti-inflammatory when tested against selected microorganisms and food materials samples [19;

43; 44]. Numerous previous studies endorsed present study results, where GC-MS analysis of *P. betle* reported the presence of phenolic compounds reported as major compounds [45; 46; 47; 48].
On the other hand, GC-MS analysis of the methanolic leaf extract of *P. odorata* showed the predominant presence of terpenes, aliphatic compounds like nonanal, hexadecanal and β-Caryophyllene. Terpenes aldehydes have a wide range of pharmacological activities [49]. Earlier studies reported that *P. odorata* has a high level of essential oils, like aliphatic aldehydes [50; 51]. Various previous studies highlighted the predominant presence of terpenes aldehydes or terpenoids from leaf extract of *P. odorata* [49; 50; 51;52]. The essential oils like terpenes aldehyde and aliphatic compounds present in *P. odorata* are assumed to be responsible for antimicrobial, antioxidant, and anti-inflammatory activities [51; 53].
The quantification of major secondary bioactive compounds is important to evaluate the therapeutic potential of the selected natural medicinal product [54]. High-performance liquid chromatography (HPLC) is a frequently used technique for rapidly determining secondary bioactive compounds [55]. In the current study, using the HPLC technique, eugenol and quercetin were successfully quantified from the methanolic leaf extract of P. betle and P. odorata. The estimated content of eugenol and quercetin bioactive compounds were 56.58 and 22.68 mg/g, respectively. Furthermore, the content of the standard compound in 10 g dried leaves of *P. betle* and *P. odorata* were 86.28 mg of eugenol and 35.72 mg of quercetin. Current study results showed enhanced quantification of eugenol and Quercetin from *P. betle* and *P. odorata*, respectively, compared to the quantification amounts reported in earlier studies [19; 28; 56].
The quantitative and qualitative biosynthesis of secondary metabolites was significantly affected in those medicinal plants which absorb heavy metals when grown in polluted environments [57]. On the other hand, heavy metal contamination is one factor responsible for the toxicity caused by medicinal plants and their by-products [57; 58]. The occurrence of heavy metals should be detrimental to health even in their trace amount [59]. In the current study, lead, arsenic, and cadmium were not detected in the methanolic leaf extract of *P. betle* and *P. odorata*. These results parallel [28], where cadmium, arsenic, mercury, and lead were not detected in the methanolic extract of *Persicaria odorata*/ *Polygonum minus* leaves.

Acute Toxicity Study:

Toxicity studies are performed to assess the safe use of medicinal plants. The primary purpose of these studies is to evaluate and establish the possible adverse effects caused by phytochemicals, especially concerning certain doses. The current study was executed to estimate the limit dose acute oral toxicity of methanolic leaf extract of *P. betle* and *P. odorata* in target species, broiler chickens.
The current limit dose toxicity mainly estimates behavioural change, body weight gain, any spontaneous change in feeding and drinking patterns, and mortality. Any hesitation in the feeding and drinking pattern indicates that the metabolism of animals undergoes some changes [60]. Except for the first 8 h, the limit dose administration of methanolic leaf extract of *P. betle* and *P. odorata* did not cause any irregularity in experimental broiler chickens' feeding and drinking patterns. Normal behavioural signs were observed in the birds, either gavaged with a low dose of 500 mg/kg or a high dose at the rate of 2000 mg/kg body weight for the observation period of the first 48 hrs. Also, the same was noted for the entire study, up to 14 days. Furthermore, no mortality was noted throughout the study period. Any rapid decrease or increase in the body weight of experimental birds is an important sign of toxicity. In the present experiment, the body weight gain of extract-treated birds was normal. In addition, there was no significant difference in BWG, FI, and FCR compared to the control group on day 7 and day 14 and during the overall period from day 1 to day 14 of the experiment. The presence of secondary metabolites like phenols (eugenol) and flavonoids (quercetin) positively influenced the birds' health; these secondary bioactive compounds are antioxidant, anti-inflammatory, and potent antimicrobial [19; 51.
Investigating blood parameters in experimental animals is a significant measure to estimate the adverse effects of any tested compound or medicinal plant extracts. Furthermore, it may also be used

as a tool to evaluate the physiological and pathological status of experimental animals [61]. The present study showed that varying doses (500, 1000, and 2000 mg/kg) of methanolic leaf extract of *P. betle* and *P. odorata* had no harmful effects on the haematological indices of experimental birds; moreover, their values were recorded within the normal range [62]. No significant ($p < 0.05$) differences in haematological indicators between extract-treated and control birds were existed. Furthermore, the normal haematology indices designated the adequacy of nutrients and the normal health status of tested/ treated birds.

Serum biochemical indices highlight the metabolic and functional status of nutrients in the animals and indicate intrinsic and extrinsic biochemical changes [63; 64]. The liver is a vital organ of living organisms. Its predominant function is detoxifying, metabolising, and eliminating endogenous and exogenous toxic substances [65; 66]. Toxicity of the liver could be determined by measuring the serum activity of ALP, AST, and ALT.

The AST and ALT activity should be considered diagnostic tools to measure hepatotoxicity [67; 68]. The increased activity of AST and ALT in individuals indicated pathological manifestation or toxicity, predicting liver injury or its impairment [68; 69; 70]. In the current study (except for AST), ALP and ALT serum concentrations were not significantly different between the extract-treated and control group birds. However, the serum concentration of AST was lower ($p < 0.05$) in birds dosed at a rate of 2000 mg/kg of *P. betle* and *P. odorata* compared to the control group of birds. This decreased activity of AST indicated the hepatoprotective effect of *P. betle* and *P. odorata*. Previous studies reported the hepatoprotective properties of *P. betel* leaves [71;72]. The *P. betle* possessed secondary bioactive compounds, including eugenol, assumed to be responsible for hepatoprotective activity, as eugenol can modulate oxidative stress by limiting the inflammatory cytokines [63;73; 74]. On the other hand, *P. odorata* possesses flavonoids, including quercetin, as secondary metabolites, which, being a strong antioxidant, are assumed to be responsible for hepatoprotective activity [22; 51; 75].

Serum proteins, including Total protein (TP), albumin, and globulin, are synthesised primarily in the liver; the concentration of serum proteins indicates the functional status of hepatocytes. Hence, a decline in the serum concentrations of proteins might result in hepatic insufficiency, malnutrition, and immune deficiency [76]. Furthermore, the normal serum concentrations of proteins highlighted the better health status of birds. In the current study, the total protein (TP), albumin, and globulin of experimental birds were recorded in the normal range.

Serum cholesterol levels and triacylglycerols indicate individuals' lipid metabolism [77]. On the other hand, electrolyte balance plays a vital role in the acid-base balance of individuals and is key to the performance of broiler birds. Thus, electrolyte imbalance might alter broiler birds' acid-base balance and metabolic functions [78]. Present study results showed no significant differences in the serum concentrations of triacylglycerols, cholesterol, and electrolyte balance between extract-treated and control group birds.

Kidney function is one of the important aspects of measuring possible toxicity. It is an essential vital organ prone to injury due to metabolic dysfunctions triggered by any endogenous or exogenous substance [79]. The serum concentrations of urea and creatinine are indicators of kidney function. The increase in serum creatinine level designates the decreased glomerular filtration that might result in kidney impairment [80]. In this study, the serum concentration of urea was decreased ($p < 0.05$) in extract-treated groups of *P. betle* (1000 and 2000mg/kg) and *P. odorata* (2000 mg/kg) compared to the control group. Meanwhile, serum concentration of creatinine decreased in extracted treated group *P. betle* (2000 mg/kg) in comparison with the control group. These results demonstrated that the methanolic leaf extract of *P. betle* and *P. odorata* had no adverse effects on the kidney function of birds. As *P. betle* and *P. odorata* possess antioxidant and anti-inflammatory properties, their secondary metabolite, including eugenol and quercetin, had ameliorated effects on renal injuries [17; 51; 81; 82; 83]. In a toxicity study, [84] reported that eugenol had hepatoprotective, antioxidant, and renoprotective properties. Similarly, [85] designated that quercetin had the therapeutic potential to ameliorate kidney injury by modulating macrophage polarisation.

Any increase or decrease in internal organs' weight in response to dietary substances indicates toxicity [86]. In the present limit dose toxicity study, no macroscopic changes like injury, swelling, hypertrophy, or atrophy were observed in the organs of experimental birds. Also, no significant differences were noted in the internal organs' weights of extract-treated birds and the control group. These results indicated that the limit dose administration of methanolic leaf extract of *P. betle* and *P. odorata* showed no harmful effect on internal organs and their weights. In addition, the histo-morphological examination of vital organs confirmed and supported the current findings. Histopathological examination designated normal histomorphological characters of the tissues of selected organs. Furthermore, no mortality was recorded in extract-treated birds till the entire study of 14 days. The present findings are in line with [28], where limit dose administration of methanolic extract of *P. odorata*/ *P. minus* leaves at a dose rate of 2000 mg/kg body weight did not exhibit any toxicity, and there was no mortality in extract-treated rats.

In the current study, the limit oral dose of methanolic leaf extract of *P. betle* and *P. odorata,* even at 2000 mg/kg, did not exhibit any toxicity or mortality. Hence, the LD_{50} value of the tested extracts was greater than 2000 mg/ kg.

Conclusions

The limit dose acute toxicity was performed to evaluate the safety of methanolic leaf extract of *P. betle* and *P. odorata* in broiler chickens. General and behavioural signs were found to be normal in birds up to a maximum dose of 2000 mg/kg body weight of experimental birds. There was no difference in the haematological blood indices and internal organ weights of experimental birds. In addition, some of the serum biochemistry parameters were improved by selected herbs extracts. Furthermore, no mortality was recorded during the 14-days study period. Based on current results, the methanolic leaf extracts of *P. betle* and *P. odorata* were considered safe up to 2000 mg/kg in broiler chickens. Further, in-feed studies of *P. betle* and *P. odorata* are needed to establish their safe inclusion doses as alternative feed additives for sustainable broiler chicken production.

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