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Acute toxicity profiling of medicinal herb *Ardisia elliptica* leaf extract by conventional evaluations and proton nuclear magnetic resonance (NMR) metabolomics

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ABSTRACT

Background and aim: Interest in the safety of herbal medicine is growing rapidly regarding knowledge and challenges in natural products. Hence, this study aimed to reveal the toxicological profile of *Ardisia elliptica*, a traditional medicinal plant used in the treatment of various illnesses.
Experimental procedure: Acute toxicity study was performed on female and male Sprague Dawley rats with a single oral administration of 2000 mg/kg BW of 70% ethanolic *A. elliptica* leaf extract, using a combination of conventional investigations and ^{1}H-NMR-based metabolomics approaches.
Results: Physical, hematological, biochemical, and histopathological assessments demonstrated the usual rat profile, with no mortality and delayed toxicity 14 days after administration. ^{1}H NMR serum metabolomics depicted similar metabolites between normal and treated groups. Nevertheless, ^{1}H NMR of urinary metabolomics revealed perturbation in carbohydrate, amino acid, and energy metabolism within 24h after extract administration, while no accumulation of toxic biomarkers in the collected biological fluids on Day 14. A minor gender-based difference revealed the influence of sex hormones and different energy expenditure on response to extract treatment.
Conclusion: This study suggested that 2000 mg/kg BW of 70% ethanolic *A. elliptica* leaf extract is considered as safe for consumption and offered a comprehensive overview of the response of physiological and metabolic aspects applicable to food and herbal product development.

Taxonomy

Traditional herbal medicine, natural products analysis, blood analysis, histopathology, behavioural toxicity, metabolomics.

1. Introduction

The use of herbal medicine dates back to ancient times and is continually practiced to date. *Ardisia elliptica* Thunb., locally known as "mata pelanduk", lán yǔ shù qǐ or Shoebutton ardisia, belongs to the family Primulaceae. It is an underutilized evergreen shrub or small fruiting plant native to Southeast Asia, especially Malaysia and other tropical areas in Taiwan, China and India, which is normally neglected by commerce.[1] However, in provincial areas, *A. elliptica* has an extensive history of traditional usage as a medicinal herb and introduced as a supplement to main diet. Its foliage is traditionally consumed as a salad in the Malay Peninsula,[2] while its decoction is used as a remedy for various illnesses, such as retrosternal pains, heartburn,[3] scabies, and intestinal worms.[4] Moreover, the edible fruits are used as folk medicine to relieve diarrhea with fever in Thailand,[5] whereas the bouillon of crushed roots is applied as a remedy in venereal diseases such as gonorrhea.[6]

There is a shift of focus to traditional medicine or natural products as

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List of abbreviations

^{1}H NMR	proton nuclear magnetic resonance
ALP	alkaline phosphatase
ALT	alanine transaminase
ANOVA	one-way analysis of variance
AST	aspartate transaminase
BW	body weight
HMDB	human metabolome database
LD_{50}	50% lethal dose
PCA	principal component analysis
PLS-DA	partial least squares discriminant analysis
SD	Sprague Dawley
TCA	the citrate cycle

remedies, accompanied by increasingly bold health claims. However, the belief that natural products are relatively safe is the key reason for overlooking the potential dangers in these traditional health foods.[7] The lack of scientific toxicological data hinders the safety evaluation of natural products. Therefore, it is vital to include animal toxicity studies in the understanding of active principles of plant materials and their mechanisms of actions in an organism.

Acute toxicity investigation usually acts as the initial screening step in the assessment and evaluation of toxic characteristics of substances.[8] Information regarding the mode of toxic action deals with dose selection, possible targeted organ determination, pharmacological aspects, clinical suitability, and any treatment-related effects.[8] Though the diverse biological activities and phytoconstituents of *A. elliptica* have been reported,[9,10] the information regarding safe prescription methods and presence of potential toxicants are still scarce. To date, only one study has reported toxicological data on *A. elliptica*, specifically affirming the safety and suitability of the fruit extract for consumption.[11]

Apart from the conventional parameters of toxicity evaluation, modern technology has been employed extensively to investigate the endogenous biological metabolites present in various model systems in recent years.[12] In this light, metabolomics provides a platform to study the metabolic variation in the body throughout the experiment. It is a powerful approach to the systematic identification and quantification of a great number of endogenous metabolites, which reflected the metabolic status of the body.[13] The metabolomic profile discloses a comprehensive overview of any abnormal deviation or presence of toxic biomarkers that could possibly affect the biological system, which is essential to the understanding of modes of action prior to physiological and pathological injuries and beneficial over conventional procedures.[14] Thus, the implementation of metabolomics in toxicology strengthens the safety evaluation of natural products.

In search of toxicological data on *A. elliptica*, owing to its high total phenolic content, strong antioxidant and anti-hyperglycemic activities,[15] the present study aimed to evaluate the acute toxicity profile of 2000 mg/kg BW of 70% ethanolic *A. elliptica* leaf extract in a rat model. A combination of conventional methods, including physical, hematological, biochemical, and histopathological assessments, together with a proton nuclear magnetic resonance (^{1}H NMR)-based metabolomics approach, were employed in this study to investigate the effect of single oral administration of *A. elliptica* leaf extract on physiological and metabolic responses.

2. Materials and methods

2.1. General materials required for the experiment

Standard (702) rat pellets were purchased from Gold Coin, Malaysia. Blood vacutainers including ethylenediaminetetraacetic acid (EDTA), plain red tubes (BD Vacutainer®), sterile syringes (1, 3, and 5 mL), needles (25G and 27G, 5/8" and 1") (Terumo Corporation), urine containers, and 16G ball-tipped stainless steel oral gavage needles (Harvard Apparatus, US) were prepared for administration, urine, and serum collection. Formaldehyde solution (37%), absolute ethanol, deuterated deuterium oxide (D_2O), non-deuterated potassium dihydrogen phosphate (KH_4PO_4), trimethylsilyl propionic acid-d4 sodium salt (TSP), and sodium azide (NaN_3) were purchased from Merck (Darmstadt, Germany).

2.2. Plant extract sample preparation

A. elliptica was harvested in University Agricultural Park in Universiti Putra Malaysia (UPM), Malaysia at geographic coordinates 2°59′27.4"N 101°42′49.7"E. The plant was identified by an in-house botanist, Dr Mohd Firdaus Ismail in the Biodiversity Unit, Institute of Bioscience, UPM with herbarium voucher number MFI 0054/19. The leaves were subjected to air drying under shade prior to grinding into a fine powder using a laboratory grinder (Waring Commercial, Torrington, CT, USA). The ground leaves were then extracted using 70% ethanol through ultrasound-assisted extraction with a ratio of 10 g sample to 100 mL solvent. The mixture was sonicated for 1 h at a controlled temperature (26°C–40°C) with a frequency of 53 kHz (Fisher Scientific, Waltham, MA, USA). The extracts were then filtered with Whatman filter paper No.1, vacuum concentrated, freeze-dried, and stored at 4°C for further study.

2.3. Animal selection and acute oral toxicity study

A total of 24 clinically healthy Sprague Dawley (SD) rats (12 female and 12 male), weighing between 180 and 230 g, aged 6–8 weeks, and purchased from Takrif Bistari Enterprise, Malaysia were selected for an acute toxicity experiment. Ethical approval was obtained from Universiti Putra Malaysia Institutional Animal Care and Use Committee (UPM/IACUC/AUP-R011/2019). All animals were housed under standard environmental conditions at a temperature of 24°C–26°C and supplied with food pellets and water *ad libitum*. Rats were acclimatized for 1 week prior to the experiment.

An acute toxicity study was performed according to the OECD 423 guidelines.[16] Generally, all experimental rats were divided into normal and treated groups (n = 6). The treatment group received a single dose of 2000 mg/kg BW of 70% ethanolic *A. elliptica* leaf extract dissolved in 0.03% carboxymethyl cellulose (CMC) through oral gavage. The normal group, given only 0.03% CMC solution, served as a control. Close monitoring was conducted for the first 6 h after extract administration to examine any obvious toxic symptoms in behavior, breathing, and posture, while regular observations were conducted daily for 14 days afterwards for any signs of distress and mortality. All experimental animals were euthanized at the end of study for organ collection and other analysis.

2.4. Physical assessment

The body weights were recorded on Days 0, 4, 7, 10, and 14, while the food and water consumptions were obtained on Days 2, 5, 8, and 11. Vital organs such as the liver, kidneys, pancreas, spleen, and gonads were collected and weighed after sacrifice at the end of the study.

2.5. Hematological and biochemical analysis

Concerning animal welfare, blood collection was only performed at the beginning and the end of the experiment through intracardiac puncture under anesthesia. Serum collected on Day 0 acted as the study baseline for NMR analysis. On Day 14, blood collected in EDTA tubes was subjected to a complete blood profile analysis, whereas the collected serum was subjected to both NMR and biochemical analyses, including

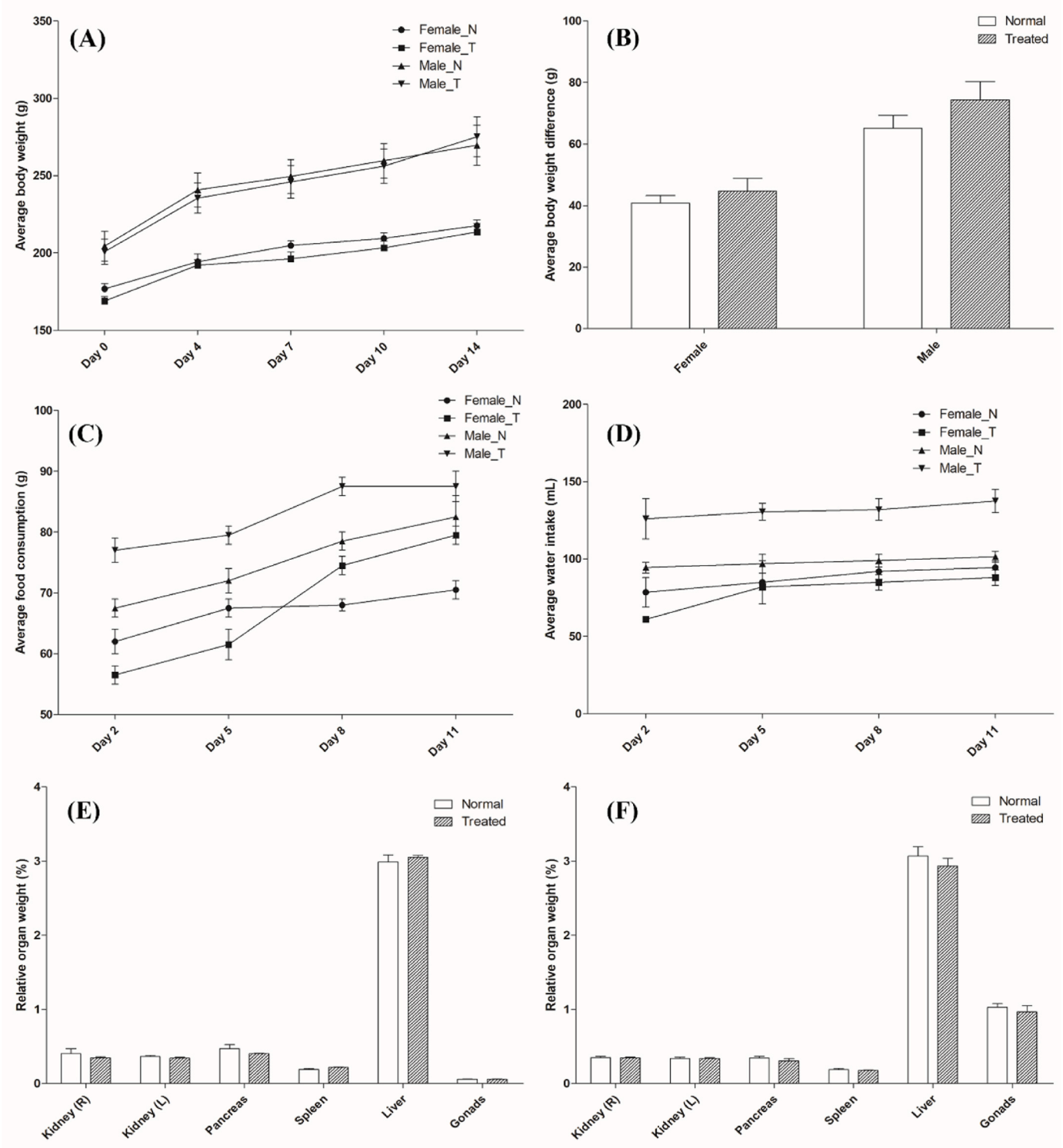

Fig. 1. Physical assessments of acute toxicity of *A. elliptica* extract, (A): Average body weight, (B): Average body weight difference, (C): Food consumption, (D): Water intake, (E): Female rats' relative organ weight, and (F): Male rats' relative organ weight; N: Normal, T: treated rats.

liver and renal function tests at the veterinary laboratory services unit, Faculty of Veterinary Medicine, UPM.

2.6. Histopathological analysis

All experimental rats were euthanized, and vital organs were collected. All organs were grossly inspected and then fixed in 10% formalin for 48 h prior to histopathological analysis at the veterinary laboratory services unit, Faculty of Veterinary Medicine, UPM. All tissues were subjected to hematoxylin and eosin staining for microscopy examination using a light microscope at 100X magnification (Zeiss Axiovert 25, Jena, Germany).

2.7. ^{1}H NMR serum and urinary sample preparation and spectra processing

Sample preparation of biological fluids for NMR acquisition was conducted according to previously described procedures, with relevant modifications.[17]

The serum samples collected were aliquoted into sterile microcentrifuge tubes and stored at −80°C prior to analysis. For ^{1}H NMR serum sample preparation, 200 μL of the thawed sample was combined with 400 μL of D_2O buffer containing KH_2PO_4 and 0.2% of TSP at pH 7.4 and subjected to ^{1}H NMR analysis using a Varian INOVA 500 MHz NMR spectrometer (Varian Inc., Palo Alto, CA, USA). A pre-saturation (PRESAT) experiment consisting of eight scans with an acquisition time of 41 s was applied to all samples to suppress the residual peak signal of water. Then, a Carr-Purcell-Meiboom-Gill pulse sequence with a T2 relaxation measurement was performed to suppress the high protein signal, with 128 scans and an acquisition time of 8 min and 12 s.

Urine samples were collected on Days 0, 1, and 14 by placing animals individually in metabolic cages with a urine collector containing 0.1% sodium azide, which acts as an anti-microbial agent. The urine was then aliquoted to sterile microcentrifuge tubes and stored at −80°C prior to analysis. For ^{1}H NMR urine sample preparation, 400 μL of the thawed sample was combined with 200 μL of D_2O buffer containing KH_2PO_4 and 0.1% of TSP at pH 7.4 and subjected to ^{1}H NMR analysis. The ^{1}H NMR spectra of urine were acquired with 64 scans, a spectral width of −2.00 to 14.00 ppm, a relaxation delay of 2.0 s, and an acquisition time of 4 min and 29 s.

The acquired NMR spectra were then subjected to manual baseline correction and spectral phasing, scaled to TSP as an internal standard. The spectral region between δ0.50 and 10.00 was binned with a spectral width of δ0.04 using Chenomx NMR software version 8.2 (Edmonton, AB, Canada). In urinary spectra, signals at δ5.57–5.95 (urea) and δ4.70–5.04 (water) were excluded, while in serum spectra, the signals δ4.71–4.97 (water) were excluded from the analysis.

2.8. Statistical and multivariate data analysis

The data collected from physical, hematological, and biochemical assessments were performed by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test to determine the significance of differences in the variables at a confidence level of 95% between groups. Statistical analyses were conducted using MS Excel 2013 (Microsoft, Redmond, WA, USA) and IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA). The processed NMR data were subjected to multivariate data analysis using Pareto scaling method in SIMCA-P software version 14.1 (Umetrics, Umeå, Sweden).

3. Results and discussion

3.1. Physical and behavioral assessments

The lethal dose (LD_{50}) of *A. elliptica* extract could not be determined, as no lethality was observed in any group of animals throughout the experiment. The LD_{50} of 70% ethanolic *A. elliptica* leaf extract is thus greater than 2000 mg/kg BW, which places the extract in the Globally Harmonized System Category 5 (LD_{50} = 2000–5000 mg/kg BW).[16] Furthermore, gross observations demonstrated that the tested animals did not produce any signs of distress or significant changes in behavior, breathing, and nervous responses during the first 6 h of administration. There was a gradual increment with no significant difference in body weight (Fig. 1A and B), indicating normal body metabolism and no occurrence of toxic effects following administration. Normal food and water consumption patterns (Fig. 1C and D) also indicated that administration of the extract did not affect the animals' appetites.[13] Generally, no morbidity, mortality, or toxic effects were observed after single-dose administration of the extract.

Table 1
Hematological and biochemical analysis on the acute toxicity effect of *A. elliptica* extract.

Parameter	Unit	Male		Female	
		Normal	Treated	Normal	Treated
Hematological analysis					
Red blood cells (RBC)	x10^{12}/L	7.53 ± 0.41	7.54 ± 0.75	8.23 ± 0.56	8.50 ± 0.37
Haemoglobin (Hb)	g/L	165.00 ± 4.53	166.83 ± 13.53	170.20 ± 12.38	175.40 ± 5.32
Packed cells volume (PCV)	L/L	0.42 ± 0.01	0.42 ± 0.03	0.44 ± 0.03	0.45 ± 0.03
Mean corpuscular volume (MCV)	fL	56.20 ± 2.17	55.17 ± 3.19	53.60 ± 1.52	53.00 ± 0.71
Mean corpuscular haemoglobin concentration (MCHC)	g/L	391.00 ± 7.55	402.17 ± 4.02	386.80 ± 11.35	390.20 ± 10.67
White blood cells (WBC)	x10^9/L	11.04 ± 2.38	9.48 ± 3.09	11.66 ± 2.93	11.62 ± 4.22
Band neutrophils	x10^9/L	0.11 ± 0.03	0.10 ± 0.03	0.12 ± 0.03	0.12 ± 0.04
Neutrophils	%	18.80 ± 4.60	17.67 ± 2.34	21.20 ± 1.10	17.60 ± 2.61
Lymphocytes	%	73.00 ± 5.34	75.17 ± 2.56	70.40 ± 1.14	75.00 ± 3.46
Monocytes	%	5.60 ± 0.55	5.17 ± 0.41	6.00 ± 0.00	5.40 ± 0.90
Eosinophils	%	1.60 ± 0.55	1.00 ± 0.00	1.40 ± 0.55	1.00 ± 0.00
Basophils	%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Platelets (PLT)	x10^9/L	1497.40 ± 75.83	1235.50 ± 567.73	1268.20 ± 318.61	1412.60 ± 297.96
P. Pro	g/L	64.80 ± 1.10	66.67 ± 3.93	60.80 ± 4.15	62.40 ± 3.58
Icterus I.	U	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00
Biochemical analysis					
Liver function tests					
Alkaline phosphatase (ALP)	U/L	120.00 ± 33.58	138.80 ± 25.08	240.80 ± 28.48	221.20 ± 25.88
Aspartate transaminase (AST)	U/L	123.40 ± 33.71	110.40 ± 14.17	146.00 ± 32.32	138.00 ± 38.94
Alanine transaminase (ALT)	U/L	32.20 ± 6.38	33.00 ± 6.00	42.20 ± 4.97	42.80 ± 4.76
Total bilirubin	mmol/L	1.22 ± 0.40	1.36 ± 0.31	1.50 ± 0.42	1.58 ± 0.49
Kidney function tests					
Creatinine	mmol/L	45.80 ± 6.30	48.50 ± 7.77	39.80 ± 2.05	43.40 ± 3.36
Urea	mmol/L	6.70 ± 1.06	8.82 ± 1.65	7.76 ± 1.54	7.08 ± 1.54

Values are expressed as mean ± standard deviation. No significant difference between normal and treated groups for both male and female rats ($p > 0.05$).

3.2. Hematological and biochemical analyses

Blood profiling is vital to the evaluation of normal body homeostasis and physiological functions, especially in the case of differential white blood cells counts, to investigate any occurrence of allergic and inflammation events due to the test substance administration.[18] As shown in Table 1, there was no significant difference between the normal and treated groups in any of the hematological parameters in either female or male rats, including RBC, Hb, PCV, MCV, and MCHC, indicating normocytic and regular erythrocytes and normal hemoglobin proportions.[19] Furthermore, no significant alterations were observed in WBC, PLT counts, P. Pro, and the icterus I., demonstrating a normal innate immune system and the absence of stimulated lymphopoiesis.[19] In view of the first-line mechanism of immune defense, the leukocyte

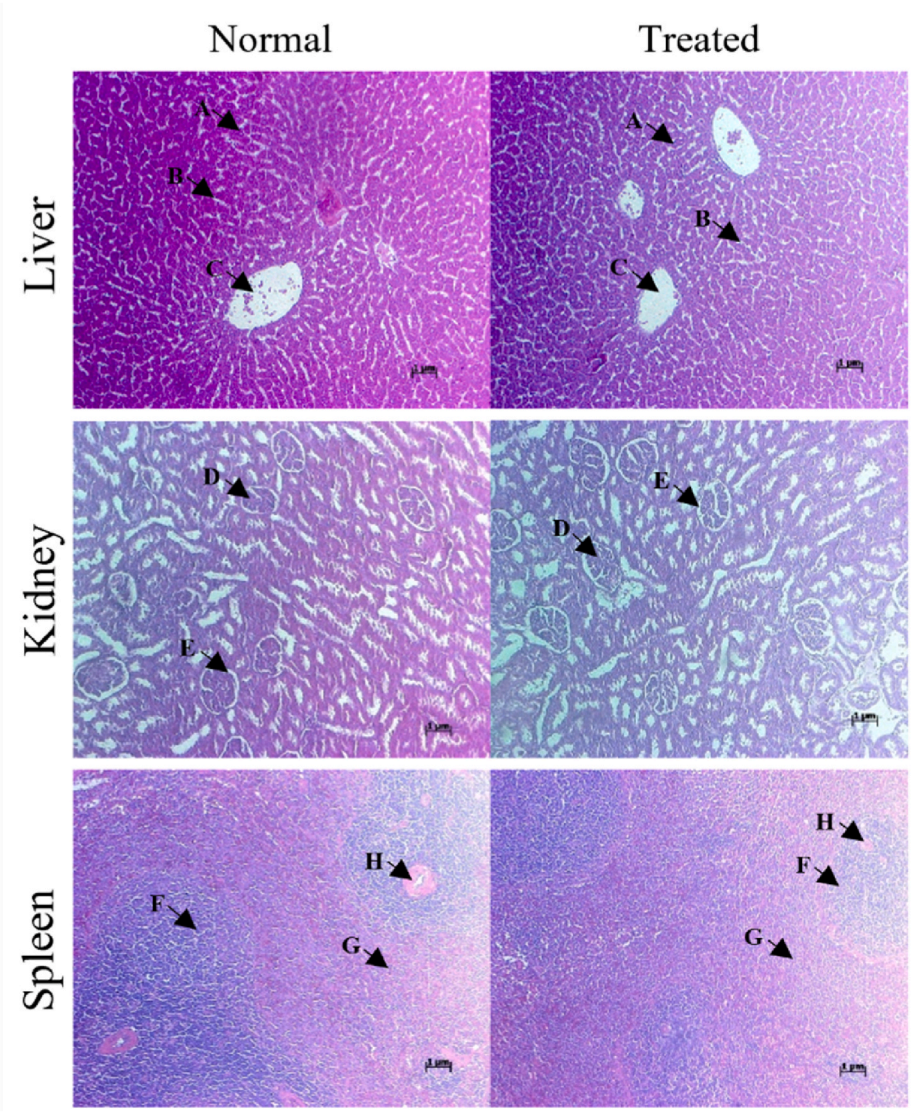

Fig. 2. Tissue cross section of representative normal and treated rats of liver, kidney and spleen (H&E staining, 100X); A: hepatocytes, B: sinusoid, C: hepatic central vein, D: glomerulus, E: Bowman's capsule, F: white pulps, G: red pulps, H: central artery.

subtypes were present in normal proportions of the circulating blood as lymphocytes (60%–89%), neutrophils (3%–20%), monocytes (1%–10%), basophils (0.5%–1%), and eosinophils (1%–5%).[18] This result indicated that there were no delayed allergic or inflammatory reactions upon extract administration.

Concerning effects on liver and kidney functions, biochemical analysis revealed hepatocellular and renal markers indicating possible damage to these organs. The liver is a complex organ that functions in macronutrient metabolism and detoxification.[20] Abnormal increases in liver enzymes such as ALP, AST, and ALT from the hepatocyte cytosol into the circulating blood are indications of liver injury in the acinar zone, changes in hepatocellular membrane permeability, and hepatic inflammation.[21] Furthermore, the bilirubin level reflects liver-specific disorders.[22] Moreover, the kidney is another vital organ with endotoxin-detoxifying and xenobiotic excretion activities, where renal parameters such as creatinine and urea levels reveal possible nephrotoxicity.[23] The results in Table 1 showed that there was no significant increment in hepato and renal markers between normal and treated rats. This is in accordance with previous study demonstrating that 5 g/kg of 95% ethanolic *A. elliptica* fruit extract did not cause acute or subacute toxicity according to hematological and biochemical results.[12] Therefore, the extract administration was deemed non-toxic to normal liver and kidney function.

3.3. Histopathological analysis

Assessment of organ-to-body weight ratios are vital to evaluate any possible organ damage preceding cell and tissue morphological changes.[24] The results (Fig. 1E and F) showed no significant differences in the relative organ weights of the liver, kidneys, spleen, pancreas, testes, and ovary between normal and treated rats, indicating that there were no signs of the hyperplasia and hypertrophy usually detected in pathological conditions.[25]

Histopathology, including macroscopic and microscopic examinations, is usually used to detect any possible pathologic changes in tissue and cell morphology. Six tissues from experimental animals were analyzed, including the liver, kidney, spleen, pancreas, testes, and ovary (Figs. 2 and 3). Microscopic observations showed normal histologic sections of liver and kidney tissues demonstrating no lesions and absence of inflammations.[26] Normal proportions of red and white pulps in spleen showed normal immune response, whereas distinct islets of Langerhans observed in pancreas indicated usual digestive reactions.[25] In view of reproductive safety, gonads from female and male rats showed normal spermatogenesis and development of ovarian follicles.[27]

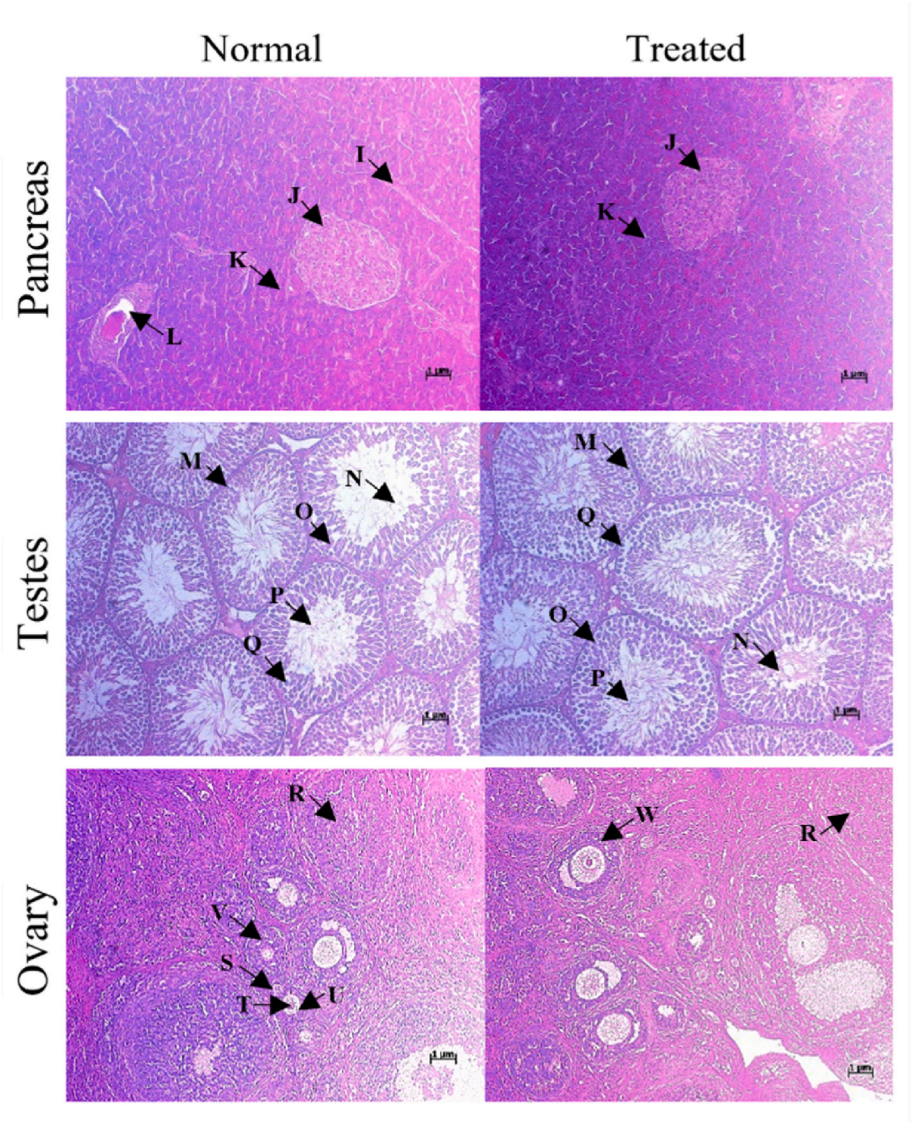

Fig. 3. Tissue cross section of representative normal and treated rats of pancreas, testes and ovary (H&E staining, 100X); I: blood vessels, J: islets of Langerhans, K: acinar cells and zymogen granules, L: interlobular duct, M: spermatogonia, N: seminiferous tubules, O: Sertoli cells, P: spermatozoa, Q: primary spermatocyte, R: corpus luteum, S: primordial follicle, T: primary oocyte, U: primary follicle, V: secondary follicle, W: Graafian follicle.

These results demonstrated the absence of treatment-induced adverse effects and signs of potential disease.

3.4. ^{1}H NMR serum metabolomics analysis

Apart from conventional toxicity evaluation techniques, ^{1}H NMR metabolomics was used to further analyze biological fluids such as serum and urine to investigate multiparametric responses in relation to toxicity. Serum collected on Day 0 and Day 14 revealed a total of 21 metabolites detected in normal and treated female and male rats (Table S1). Metabolites were identified by using the Chenomx software database, the human metabolome database (HMDB), and literature data matching.[13] There were no significant differences in visual inspection of ^{1}H NMR spectra between normal and treated rats.

Unsupervised principal component analysis (PCA) score plots were generated to illustrate biomarker variation in the sera for female (Fig. 4A) and male (Fig. 4B) rats. Based on the plots, PC1 discriminated Day 0 and Day 14 sera by 51.4% and 51.7% of the variation in female and male rats, respectively, while normal and treated rats had high similarity in endogenous metabolites for both female and male rats. The discriminated metabolites between these clusters had a higher intensity on Day 14 compared to Day 0 as shown in the loading plots. Glucose, lactate, and pyruvate are common intermediate substrates used in energy production and were anticipated to be higher in serum collected on Day 14 of the experiment, as rats approximately 10 weeks of age had higher growth and metabolic rates.[13] In accordance, certain circulating amino acids were present in higher concentrations at the end of the experiment, including glycine, which is normally obtained from the diet or synthesized via the choline biosynthetic pathway, where *N,N*-dimethylglycine is a common intermediate metabolite,[28] alanine and glutamine in gluconeogenesis, especially in the fasting state.[29] Besides, leucine, isoleucine, and valine, involved in gluconeogenesis, rate of protein synthesis, and muscle rebuilding and recovery were found to be higher on Day 14.[29] Higher rates of cellular energy metabolism in rats are associated with higher serum creatine and guanidoacetate levels,[30] whereas acetone, acetate, and acetoacetate are metabolites involved in ketogenesis where the carbohydrate source is scarce and act as alternative fuels.[13] ^{1}H NMR serum metabolomics demonstrated the absence of abnormal or toxic biomarkers observed in circulating blood, in accordance with hematological and biochemical analysis. Hence, urine

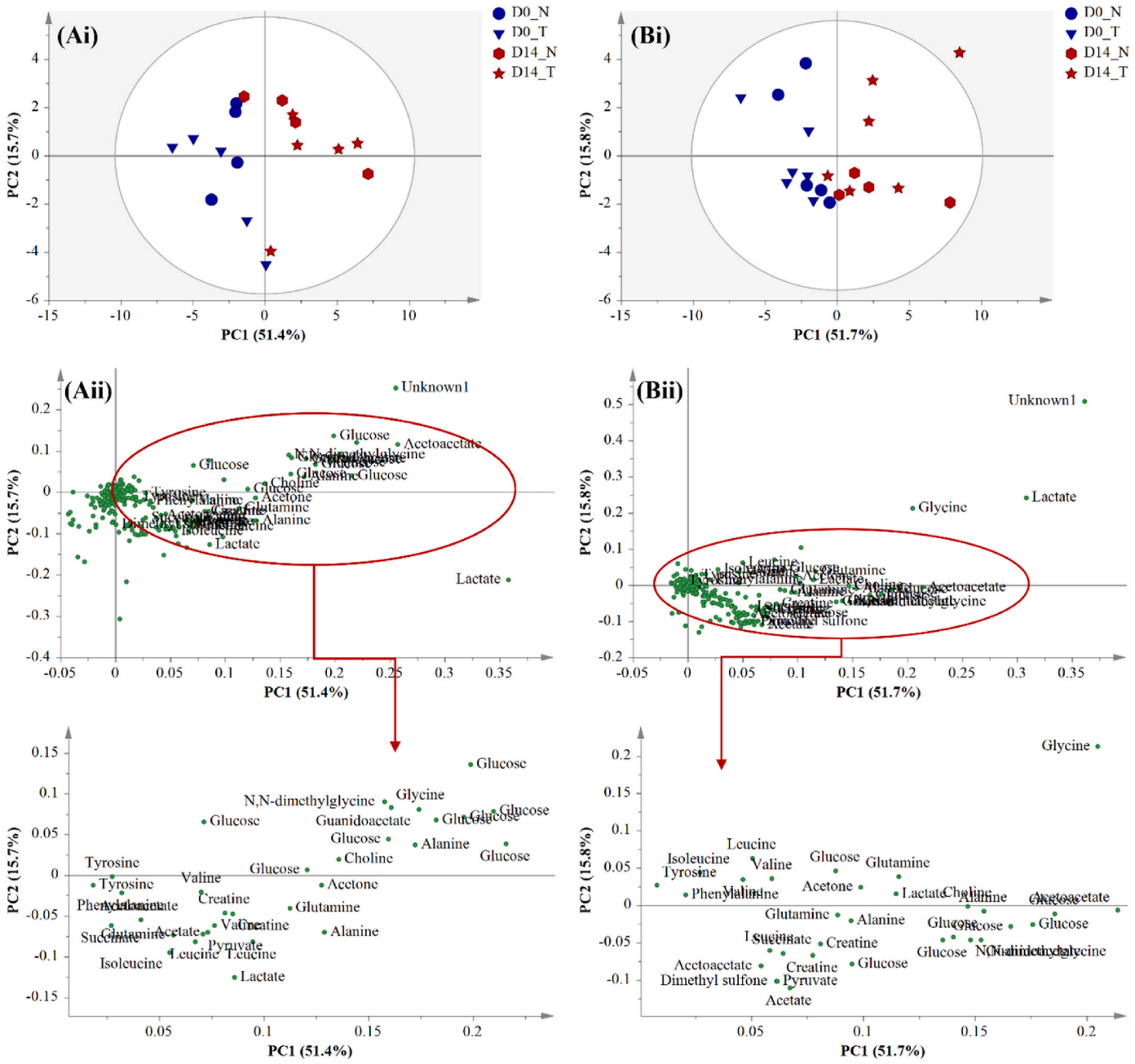

Fig. 4. PCA score plots of (Ai): female and (Bi): male rats, and corresponding loading plots (Aii) and (Bii) of serum collected on Days 0 and 14; N: Normal, T: treated rats.

collected throughout the experiment was used to further analyze and support the observations.

3.5. *^{1}H NMR urinary metabolomics analysis and suggested metabolic pathways*

Urinary excretion is a vital elimination route through which absorbed exogenous materials and endogenous waste products are eliminated from the body. Higher quantity of metabolites detected in the urinary metabolomic profile could reflect the metabolic status of the organism. Metabolites were identified on the basis of their ^{1}H NMR urine spectra collected on Day 0, the day of extract administration (Day 1), and Day 14. A total of 39 metabolites were tentatively identified in both female and male rats, including 3 unknown metabolites, which were found to contribute significantly to cluster separations (Table S2). Hence, multivariate data analysis was utilized to investigate the clustering characteristics and urinary metabolite alterations.

The overall urinary trajectory from the beginning to the end of the study was illustrated by PCA score plots (Fig. 5). Both female and male rats displayed similar clustering patterns, suggesting that the treated group on Day 1 was clearly distinguished from the normal group, while all rats on Day 14 showed the greatest variation in urinary metabolites as compared to Day 0 (pre-dosed). Based on the plots, PC1 separated Day 0 and Day 14, whereas PC2 separated the Day 1 treated group from the normal group. Normal and treatment groups were clustered together on both Days 0 and 14, indicating insignificant metabolite alteration and the absence of adverse toxicity in the rats' urinary metabolic profiles 14 days after extract administration. To ensure that no inimical metabolic effect on the rats, partial least squares discriminant analysis (PLS-DA) models were generated to further analyze the metabolite differentiation

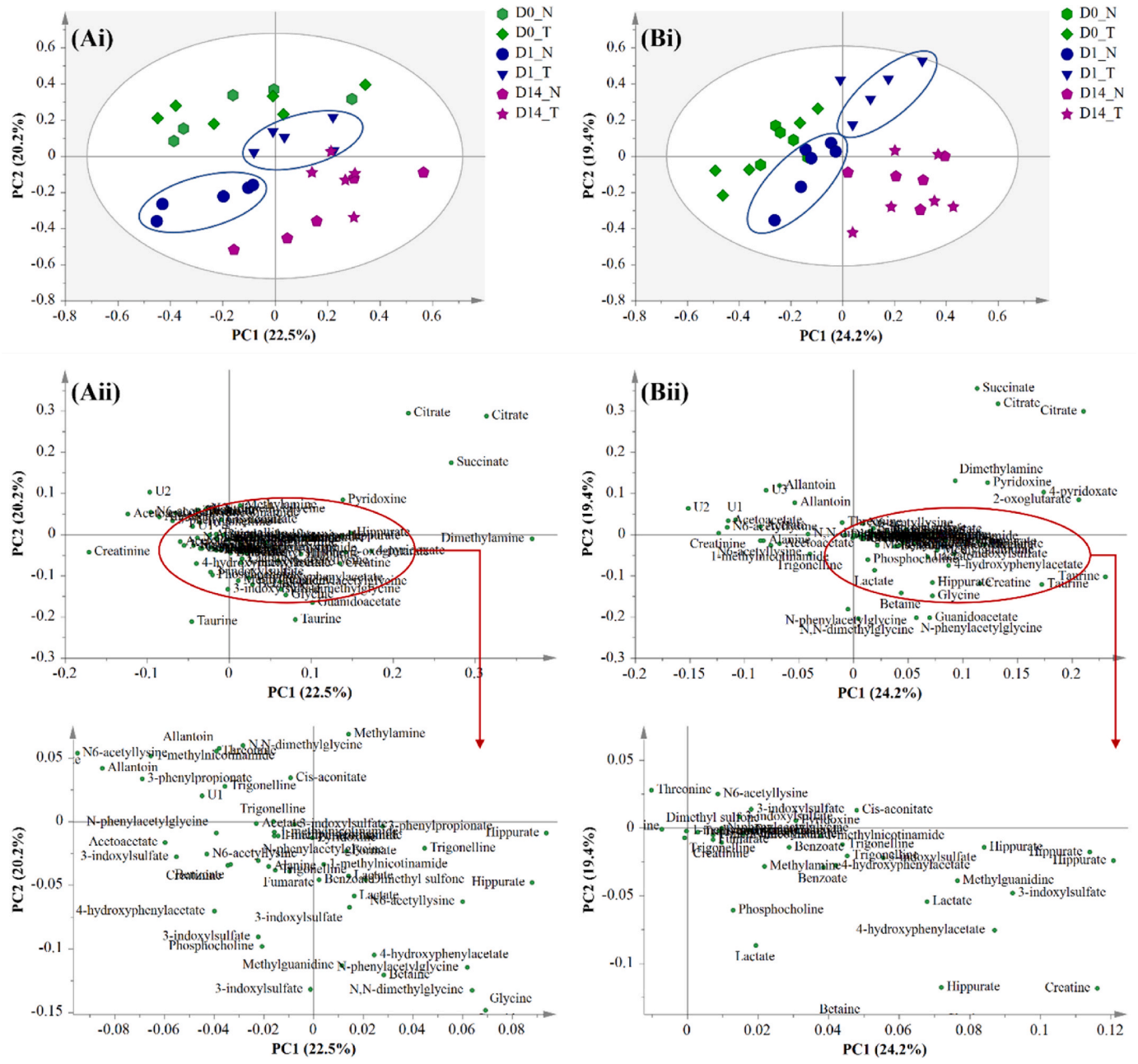

Fig. 5. PCA score plots of (Ai): female and (Bi): male rats, and corresponding loading plots (Aii) and (Bii) of urine collected on Days 0, 1 and 14; N: Normal, T: treated rats.

among the urine samples collected on Days 0, 1, and 14 from the treated group only.

Based on the PLS-DA score plots (Fig. 6Ai and Bi), PC1 separated urine collected on Day 0 from Day 14, while Day 1 was separated from Days 0 and 14 by PC2 in both female and male rats. In alignment with the PCA models, loading plots (Fig. 6Aii and Bii) revealed a more detailed outlook on metabolite variations in the same group of rats throughout the 14 days of the experiment. Metabolites in urine collected on Day 1 with higher concentrations included succinate, citrate, pyridoxine, 4-pyridoxate, 2-oxoglutarate, allantoin, and unknown metabolite 1. Nevertheless, guanidoacetate, *N,N*-dimethylglycine, *N*-phenylacetylglycine, betaine, hippurate, dimethylamine, creatine, glycine, taurine, 3-indoxylsulfate, and lactate were the main metabolites that contributed to the differentiation of Day 14. Moreover, creatinine, acetoacetate, 1-methylnicotinamide, N6-acetyllysine, threonine, and unknown metabolites 2 and 3 were identified on Day 0. Therefore, these metabolites (VIP value > 1.0) were quantified relatively, and their fold changes are summarized in Table S3. All the models were statistically verified with internal validations, permutation analysis (Fig. S1) and significant CV-ANOVA, demonstrating that the models are reliable and robust.[31]

In the understanding of linked reactions within an organism, metabolic pathways that are highly associated with the identified urinary metabolites were analyzed using the KEGG online database and are depicted in Fig. 7. Generally, a total of 7 and 6 impacted pathways were suggested by MetaboAnalyst for female and male rats, respectively. These pathways included the citrate cycle (TCA cycle); glycine, serine, and threonine metabolism; glyoxylate and dicarboxylate metabolism; taurine and hypotaurine metabolism; butanoate metabolism; synthesis and degradation of ketone body pathways; and nicotinate and nicotinamide metabolism (only in female rats), bearing an impact value higher than 0.10, suggesting that they were the most relevant pathways

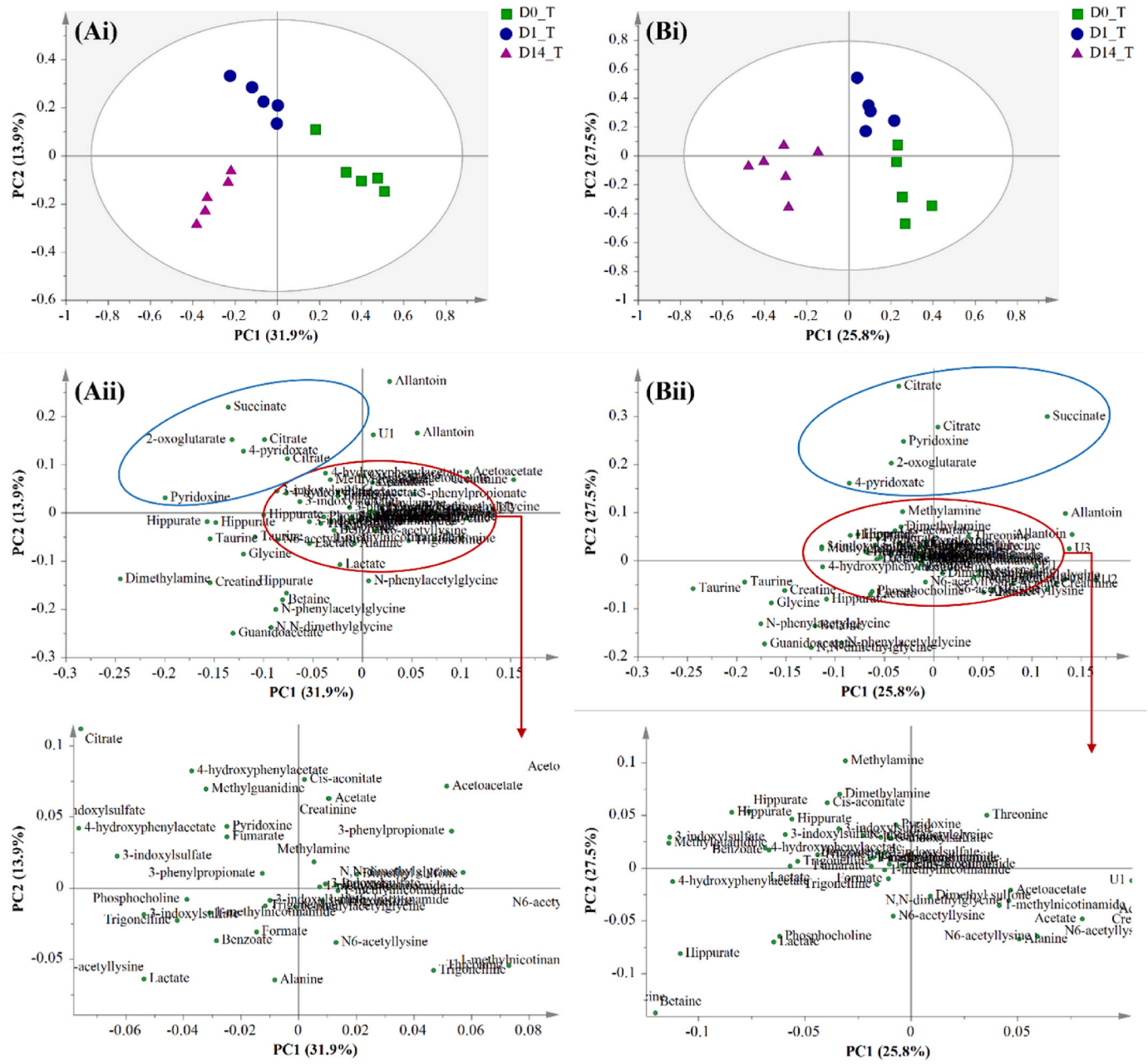

Fig. 6. PLS-DA score plots of (Ai): female and (Bi): male treated rats, and corresponding loading plots (Aii) and (Bii) of urine collected on Days 0, 1 and 14; N: Normal, T: treated rats.

involved in the metabolism of treated rats 14 days after administration.

Among all affected pathways, disturbance of metabolites associated with carbohydrate and energy metabolism was observed on Day 1, including the citrate cycle and ketone body production and degeneration. The TCA cycle is the essential metabolic pathway responsible for the oxidative degradation of macronutrients in energy production.[32] Upregulation of urinary levels of succinate, citrate, and 2-oxoglutarate indicated alterations in energy homeostasis. Succinate and citrate are weak acids and have multiple roles in biological metabolism in animals.[32] Elevation of urinary succinate and citrate levels demonstrated an increase in the metabolic activity of the rats, which aligned with a previous study showing a similar incremental pattern within 24 h after epicatechin administration.[33] In addition, acetoacetate is mainly used as an alternative source of energy and its downregulation within 24 h after extract administration suggested increased activity of aerobic respiration and improvement of energy metabolism.[34] This phenomenon was significant in treated male rats only, which might be justified by male has a greater aerobic capacity compared to female.[34]

In view of the influences of gender differences, downregulation of the urinary 1-methylnicotinamide level was significant in treated female rats only. It might be associated with the lower aerobic activity in female rats, as a higher exercise rate promoted the level of 1-methylnicotinamide.[35] Besides, 1-methylnicotinamide is a mononucleotide by-product of nicotinamide regularly converted by the kidneys. Elevation of this metabolite in urine acted as a biomarker for acute kidney injury.[36] In this light, a decrease in the level of urinary 1-methylnicotinamide demonstrated the absence of kidney injury. Moreover, reduced level of urinary threonine observed in treated female rats indicated an increased ability of liver to decompose threonine by elevating the activity of threonine dehydratase in hepatocytes. This study showed the potential presence of a relationship between sex hormones and enzymatic activity as well as nitrogen sparing in rats.[37]

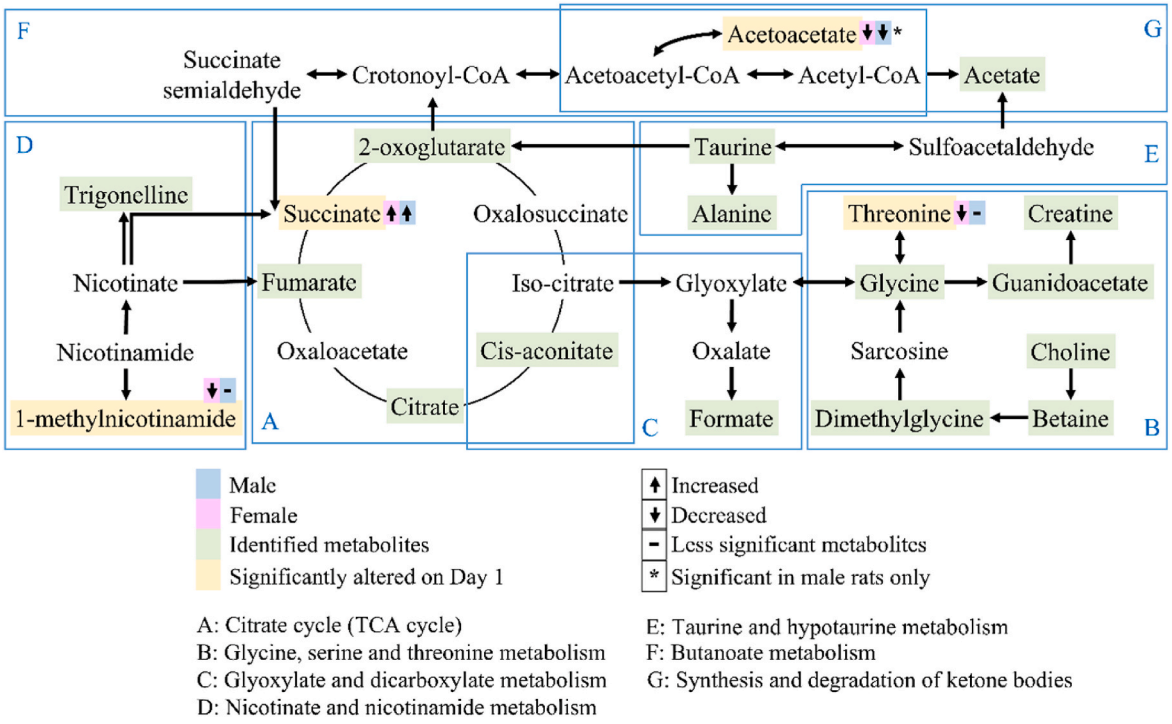

Fig. 7. The schematic diagram of the suggested metabolic pathway for the urine collected from both female and male rats.

Several significant changes in metabolites were detected on Day 1. Pyridoxine is an essential nutrient commonly obtained in food and used as a dietary supplement. 4-pyridoxate is the catabolic product of pyridoxine and is regularly excreted in urine.[38] The elevation of these metabolites portrayed the potential uptake through diet and extract administration. Interestingly, urinary N6-acetyllysine levels on Days 1 and 14 were notably lower than on Day 0. Elevation of this metabolite usually acts as a biomarker in renal diseases[38] and amino acid metabolism disorders.[39] Downregulation of N6-acetyllysine in this study demonstrated the absence of acute adverse effects of extract administration on kidney function and protein metabolism.

Additionally, metabolomics revealed the changes in urinary metabolites distinguished on Day 14. Elevation of betaine and its metabolic product *N,N*-dimethylglycine suggested enhancement of amino acid metabolism, which further metabolized in the liver to glycine.[40] In addition, hippurate, taurine, guanidoacetate and creatine were notably elevated demonstrating that metabolites in normal healthy rats change over time, especially during weeks 6–10 with distinct clusters in the urinary metabolic profile.[41] Apart from higher dietary uptake demonstrated in the female normal group compared to treated group, which stimulates an increase in urinary creatine level, the influence of sex hormones on creatine/creatine kinase regulation for energy metabolism may contribute to a greater accumulation in female normal rats.[42] Overall, the ^{1}H NMR urinary metabolic profile suggested that *A. elliptica* leaf extract did not stimulate acute toxicity events, which further justified the conventional findings and serum metabolomics analysis.

4. Conclusion

In conclusion, this study disclosed that a combination of conventional analysis and metabolomics techniques are effective in the evaluation of toxicity reactions in an organism. The findings confirmed that oral administration of 70% ethanolic *A. elliptica* leaf extract has a LD_{50} higher than 2000 mg/kg BW. The conventional analyses showed that there were no stimulated allergic reactions or abnormal detoxifying events, with normal blood and enzymatic profiles. Both serum and urinary ^{1}H-NMR-based metabolomics analysis supported the conventional findings, with suggested metabolic pathways revealing the absence of toxic biomarkers or bizarre alterations in endogenous metabolites 14 days after single administration. These results proposed the safety of *A. elliptica* leaves for consumption, which solidified the traditional usage of this plant. However, further toxicity examination should be conducted to investigate the dose- and time-related influence of *A. elliptica* on body metabolism in functional food and pharmaceutical development.

Ethics approval

The use and care of animals were reviewed for ethical usage as per Universiti Putra Malaysia Institutional Animal Care and Use Committee (UPM/IACUC/AUP-R011/2019).

The plant sample *A. elliptica* was harvested in University Agricultural Park in Universiti Putra Malaysia (UPM), Malaysia, and identified by an in-house botanist, Dr Mohd Firdaus Ismail in the Biodiversity Unit, Institute of Bioscience, UPM.

Consent for publication

All the authors consent to the publication of the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2024.02.001.

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