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Phytochemical profile and hepatoprotective activity of *Moringa oleifera* Lam leaf extract in acetaminophen-challenged mice

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ABSTRACT

Moringa oleifera leaves are rich in phytochemicals, including flavonoids and polyphenols, with reported antioxidant and hepatoprotective properties. This study evaluated the phytochemical profile of an aqueous-ethanolic extract and its efficacy against acetaminophen (APAP)-induced hepatotoxicity in mice. LC-MS/MS identified 80 metabolites, mainly flavonoids and amino acids. The extract showed moderate antioxidant activity in DPPH and FRAP assays, with a total phenolic and total flavonoid contents of 24.73 mg GAE/g DW and 2.95 mg CE/g DW, respectively. In vivo, mice given toxic APAP doses and treated with the extract (50 or 200 mg/kg) exhibited improved liver histology, reduced serum ALT and AST, suppressed CYP2E1 activity, and restored glutathione levels. Proinflammatory cytokines (IL-1α, IL-1β, IL-6, MIP-1α, MCP-1, TNF-α) and hepatic iNOS and NF-κB expression were also downregulated. Additionally, liver metabolomics revealed normalization of lipid metabolism and prostaglandin levels. These findings suggest that *M. oleifera* extract confers hepatoprotection via antioxidant, anti-inflammatory, and metabolic pathways, supporting its potential in functional food and nutraceutical applications.

1. Introduction

The recognition of plant-based natural products as valuable sources of bioactive chemicals is a longstanding phenomenon, mostly attributed to the wide range of phytochemicals they produce (Teoh et al., 2023). These natural extracts contain a wide range of bioactive constituents including alkaloids, flavonoids, and terpenoids, which exhibit diverse biological activities such as antioxidant, anti-inflammatory, antimicrobial, and anticancer effects.

Recent studies highlight the health-promoting potential of plant-derived compounds in animal and human models. For instance, basil (*Ocimum* spp.) essential oils and extracts exhibit antioxidant, antiviral, and antimicrobial activities and can inhibit human breast cancer cell viability (Al Husnain et al., 2024); frankincense extracts enriched with boswellic acids restore renal function in acute kidney injury by reducing oxidative stress and inflammation (Balgoon & Alghamdi, 2024); and lycopene from tomatoes protects the liver against chemical-induced damage by restoring enzyme activity and improving tissue histology (Hammoud et al., 2025). These findings underscore medicinal plants as valuable sources of therapeutic agents for overall health.

Among these, *Moringa oleifera* (hereafter referred to as Moringa) has gained considerable attention for its role in enhancing general health and supporting lactation, particularly during the postpartum period (Cohen-Zinder et al., 2017; Fungtammasan & Phupong, 2022; Liu et al., 2023; Rotella et al., 2023). Moringa, commonly known as the “miracle tree”, is renowned for its abundant nutritional content, including vitamins, minerals, and a range of bioactive chemicals. As a result, it is widely used in traditional medicine and applied across multiple biological systems.

Moringa leaf extract acts as a natural biostimulant in plants (Al-Muhe

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& Abdul-Wahid, 2024), while in poultry, low-level dietary supplementation enhances growth, immune function, and stress tolerance by reducing corticosterone and inflammatory responses (Al-Suwailem et al., 2024). In human and animal health models, Moringa leaf extract exhibits strong hepatoprotective and antioxidant effects against toxicants such as heavy metals and nanoparticles and has been associated with therapeutic benefits in hypertension, diabetes, and cancer (Bashir et al., 2024). Overall, Moringa functions as a biological "multi-tool", supporting growth, immune defense, and cellular protection across biological systems.

Moringa extracts possess significant antioxidant, anti-inflammatory, antimicrobial, and antidiabetic properties (Islam et al., 2021; Peñalver et al., 2022). Under normal circumstances, free radicals generated during the body's normal physiological processes will be regulated via glutathione metabolisms and antioxidant metabolites (Di Giacomo et al., 2023; Yoshikawa & You, 2024). Nevertheless, the continuous exposure to environmental contaminants, pesticides, radiation, and drugs will cause an imbalance in the endogenous antioxidant system, contributing to the build-up of oxidative stress (Burton & Jauniaux, 2011; Yoshikawa & You, 2024). Supplementation of antioxidants from external sources has been demonstrated to augment the inherent antioxidant defense mechanism by enabling the penetration of these antioxidant compounds through the gastrointestinal barrier. Preclinical evaluations have demonstrated that crude extracts of medicinal plants exhibit synergistic effects in terms of antioxidant activities, with almost no toxicity (Teoh et al., 2023). Commercialisation of bioactive compounds, particularly flavonoids, into therapeutic drugs is often hindered by the challenge of achieving bioavailability of the individual compounds in in vivo studies (Thilakarathna & Rupasinghe, 2013; Yuan et al., 2024). Hence, adopting the administration of crude extracts or a combination of bioactive chemicals might be considered an appropriate approach to address the issue of bioavailability.

Liver disease has been reported to contribute to 4% of death worldwide annually. Although virus-induced liver injury is the most common cause of liver disease, drug-induced liver injury has increased significant proportion of cases recently (Devarbhavi et al., 2023). Among the common drugs that were associated with induction of liver disease, acetaminophen (APAP) overdose was one of the leading causes that was more common and severe in women (Rubin et al., 2018). *N*-acetyl-p-benzoquinone imine (NAPQI) generated by overdose of acetaminophen depleted the glutathione in the liver cells and thus contributed to the oxidation stress and proinflammation responses led to the hepatotoxicity (Yan et al., 2018). Previous studies have proposed that decreasing liver oxidative stress (Yan et al., 2018) and inflammation (Liao et al., 2023) were the strategies that may help to ameliorate the APAP-induced liver damage based on the preclinical researches.

Natural products have been reported with potential to restore the liver function due to their antioxidant and anti-inflammatory activities, contributed by the bioactive compounds including polyphenols and flavonoids (Liao et al., 2023). Among the natural products, Moringa leaves has been recognised as a highly nutritive vegetable, which are a good natural source of antioxidant (Adejumo et al., 2012; Gao et al., 2025). Study has profiled that Moringa leave ethanol extract was reported with high diversity of phenolics (such as caffeic acid, gallic acid, p-coumaric acids) and flavonoids (catechin, rutin, quercetin and cinnamic acid) (Aly et al., 2020). These bioactive compounds may contribute to the therapeutical potential of Moringa against APAP-induced hepatotoxicity via reduction of lipid peroxidation in the rat model (Sharifudin et al., 2013). In addition, Aly et al. (2020) also reported that Moringa ethanol extract supress expression of the proinflammatory cytokines TNF-alpha and TGF-beta in the APAP-induced liver damage rat model. Although the general antioxidant and anti-inflammatory effects of Moringa ethanol extract on APAP-induced liver damage was reported, the detail mechanisms was not reported. Thus, this study provides insight into Moringa for treatment of APAP-induced liver damage throughout the cytokines profiling and metabolomics analysis.

This study aims to bridge that gap by evaluating the hepatoprotective mechanisms of Moringa ethanol extract through cytokine profiling and liver metabolomics analysis in a murine APAP toxicity model. In addition, we sought to profile the phytochemical constituents of the extract and relate them to its in vitro antioxidant capacity. This comprehensive approach will provide deeper insights into the bioactive components of Moringa and their therapeutic relevance, contributing to scientific validation and potential applications of this traditional medicinal plant.

2. Experimental

2.1. Samples acquisition

Moringa oleifera Lam. (Moringa) leave extract was acquired and processed by the manufacturer (Lot number: REXTHMO2023) (Elken Sdn Bhd, Subang Jaya, Malaysia). Briefly, dried and grounded Moringa leaves were extracted via overnight maceration in 80% of ethanol. Prior to the filtration with Whatman filter paper to recover the extract solution, a 1-h sonication (500 W, 20 kHz) was introduced to the sample mixture to facilitate cell wall disruption and increase mass transfer, thereby improving the extraction efficiency of ethanol-soluble bioactive compounds. Following, recovered extract was dried with rotary evaporator.

2.2. Sample identification

Genomic DNA was extracted from grounded Moringa leaves (Lot number: REXTHMO2023) (Elken Sdn Bhd, Subang Jaya, Malaysia) using cetyltrimethylammonium bromide (CTAB) Extraction Solution (G-Biosciences, St. Louis, MO, USA) following the manufacturer's protocol. The DNA extracted was used for PCR amplification of the ITS2 locus using ITS-2 forward (5′- ATGCGATACTTGGTGTGAAT −3′) and reverse (5′-TCCTCCGCTTATTGATATGC -3′) primers following the parameters as described by Hassan et al. (2020). After PCR cleanup, the amplified product was sent for Sanger sequencing (Apical Scientific, Seri Kembangan, Malaysia). The sequence was then compared with the NCBI GenBank using Basic Local Alignment Search Tool for nucleotides (BLASTn) to identify the species of the Moringa sample.

2.3. Radical scavenging activity (DPPH assay)

The radical scavenging activity (RSA) of Moringa leaf extract powder was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, with gallic acid as a positive control (Brand-Williams et al., 1995; Gnanaraj et al., 2017). Absorbance was measured at 515 nm using a spectrophotometer (TECAN Spark microplate reader). The results were calculated and expressed as % RSA according to the equation:

$$\%RSA = \left[\left(A_{Control} - A_{Sample}\right)/A_{Control}\right] \times 100\%$$

where A_{sample} is the absorbance (515 nm) value of the plant extract, while $A_{Control}$ is the absorbance (515 nm) value of the solvent. Detailed methodology can be found in Supplementary Information (SI: Section 2.3).

2.4. Ferric reducing antioxidant power (FRAP) assay

The reducing capacity of Moringa leaf extract powder was determined using FRAP assay (Mwamatope et al., 2020; Thaipong et al., 2006) with gallic acid as the reference standard (50–1000 μg/mL concentration). The results were presented as the concentration of gallic acid equivalent in milligrams per gram of sample dry (mg GAE/g DW). Detailed methodology can be found in SI (Section 2.4).

2.5. Total phenolic content (TPC)

TPC in Moringa leaf extract powder was determined using Folin-Ciocalteu (FC) colorimetry method as described by Mwamatope et al. (2020), with gallic acid served as reference standard (50–1000 μg/mL concentration). The results were presented as the concentration of gallic acid equivalent in milligrams per gram of sample dry (mg GAE/g DW). Detailed methodology can be found in SI (Section 2.5).

2.6. Total flavonoid content (TFC)

TFC in Moringa leaf extract was determined using the aluminium chloride colorimetric method (Zhishen et al., 1999), with catechin as the reference standard. The results were expressed as mg of catechin equivalents per g of dry weight (mg CE/g DW). Detailed methodology can be found in SI (Section 2.6).

2.7. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) profiling

LC-MS/MS profiling was performed using a Dionex UltiMate 3000 ultra-high-performance liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Thermo Scientific Q Exactive HF Orbitrap mass spectrometry system (Thermo Fisher Scientific, Waltham, MA, USA). The aqueous ethanolic extract of Moringa was re-constituted in 500 μL of MS-grade methanol and filtered with 0.22 μm polytetrafluoroethylene (PTFE) syringe filter, before LC-MS/MS profiling. Extracts were chromatographically separated via a reversed phase Kinetex F5 column (1.7 μm, 2.1 mm × 100 mm; Phenomenex, Torrance, CA, USA) as reported previously (Yong et al., 2017). Ionization was carried out using a heated electrospray ionization (HESI) probe. Chromatographic separation was performed at 35 °C with a flow rate of 450 μL/min, using water (solvent A) and 60% (*v*/v) of acetonitrile in methanol (solvent B) as the mobile phases. Furthermore, 0.1% formic acid and 1% 10 mM ammonium formate were supplemented to both mobile phases to enhance ionizations. The elution was performed using gradient, from 1% - 100% of solvent B over a period of 10 mins, which was then maintained for 3 mins. Data dependent acquisition (DDA) were performed in positive and negative modes as described in Teoh et al. (2023).

2.8. Putative metabolites identification

Acquired data were pre-processed using Compound Discoverer 3.3 (Thermo Fisher Scientific, Waltham, MA, USA), employing the default workflow for natural products analysis (Ng et al., 2024). Compound identification was subsequently performed by matching the MS/MS spectra of each feature against the mzCloud database. Features that remained unidentified were further queried against the ChemSpider database (Pence & Williams, 2010) using MS data, whereby only those with FISh score > 50 were retained.

2.9. Quantitative analysis of selected metabolites

Following the putative identification via LC-MS/MS profiling, kaempferol, quercetin, luteolin and vitexin were selected for quantitative analysis. The Moringa leaf extracts were accurately weighed and reconstituted in methanol and spiked with standard compound for respective quantitative analysis. In brief, 1 mL of a 100 mg/mL extract was mixed with 0.01–0.05 mL of 5 mg/mL standard solution. Before the analysis, the volumes of the mixtures were adjusted to 1.5 mL by adding methanol. All samples were filtered with 0.22 μm PTFE syringe filter prior to analysis via Waters ACQUITY Arc UPLC system equipped with a photodiode array (PDA) detector (Milford, MA, US). Respective chromatography separation was performed with Kinetex PS C18 column (2.6 μm, 4.6 mm × 150 mm; Phenomenex, Torrance, CA, USA), and the column temperature was maintained at 35 °C. The mobile phases were composed of water (solvent A) and acetonitrile (solvent B), and both were supplemented with 0.1% formic acid. The gradient elution for kaempferol, quercetin and luteolin was initially maintained at 10% solvent B for 5 mins, then linearly increased to 100% solvent B in 19.5 mins and maintained for 5 mins; whereas for vitexin, 10% solvent B was kept for 5 mins, then gradually increased to 35% in 7.5 mins and kept for 3 mins, followed by 100% solvent B in the next 12 mins and kept for 5 mins. Later, the column was conditioned as initial before the next injection. The acquired data was processed with Waters Empower 3 software. The results were calculated using the following formula and expressed as mg/kg of extract:

$$Conc.\,of\,flavonoid = \frac{-(x - intercept) \times concentration\,of\,spiked\,standard}{volume\,of\,sample}$$

2.10. Animals

The in vivo study was performed according to the guideline of Universiti Putra Malaysia Institutional Animal Care and Use Committee (IACUC) with the approval number (UPM/IACUC/AUP-R025/2024). A total of 30 BALB/c mice (female, 5–6 weeks old) were purchased from animal facility, Universiti Putra Malaysia and acclimatized under controlled condition (22 ± 1 °C, a 12-h light/dark cycle) with basal diet (Mouse pellet 702-P, Gold Coin Co., Limited, Malaysia) along with ad libitum access to distilled water. The mice were divided into five groups, with all except the normal control group (N) receiving acetaminophen (APAP) (Sigma, St. Louis, MO, USA) (250 mg/kg body weight) via oral gavage for 7 days to induce liver damage. After that, mice were subjected to 30 days of treatment via oral gavage, while mice under normal control and untreated groups were fed orally with distilled water. Below was the grouping,

Normal (n = 6)	: Normal control (N)
Untreated (n = 6)	: Untreated APAP-challenged mice
Moringa Low (n = 6)	: Moringa 50 mg/kg body weight (BW) treated APAP-challenged mice
Moringa High (n = 6)	: Moringa 200 mg/kg body weight (BW) treated APAP-challenged mice
Silybin (n = 6)	: Silybin 50 mg/kg body weight (BW) treated APAP-challenged mice

All samples were prepared freshly prior to usage. At the end of the treatment, the mice were euthanized under ketamine-xylazine anesthesia (100 mg ketamine and 10 mg xylazine per kg body weight). Blood samples were collected from their hearts by cardiac puncture and liver was harvested for further analysis.

2.11. Liver histopathological analysis

Liver was harvested and immersed in formalin for 24 h. The fixed tissues were embedded in paraffin, sectioning, deparaffinization, and rehydration following standard protocols followed by hematoxylin and eosin (H&E) staining. The stained liver sections were examined using bright-field optics at 40× magnification on a light microscope (Leica, Wetzlar, Germany).

2.12. Liver function tests

Serum level of liver enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) indicating liver function were analyzed using biochemical analyzer (Hitachi

Table 1
Top five BLASTn results of PCR product sequence.

Scientific Name	Max Score	Total Score	Query Cover	E value	Percentage. Identity	Accession Length	Accession
M. oleifera	263	263	100%	5e-66	100.00%	342	PQ000110.1
M. oleifera	261	261	99%	2e-65	100.00%	687	KT737754.1
M. oleifera	261	261	99%	2e-65	100.00%	687	KT737757.1
M. oleifera	257	257	100%	2e-64	99.30%	693	KT737773.1
M. oleifera	257	257	100%	2e-64	99.30%	693	KT737774.1

902 Automatic Analyzer; Hitachi, Tokyo, Japan) with reagents from Roche Diagnostics (Indianapolis, IN, USA).

2.13. Liver cytochrome P450 2E1 expression

Total protein from the fresh liver tissue was extracted using radioimmunoprecipitation assay (RIPA) buffer with phosphatase inhibitor cocktail (Roche, Indianapolis, IN, USA). Cytochrome P450 2E1 (CYP450 2E1) (Cat no: NBP1–85367) (Novus Biological, Centennial, CO, USA) protein expression level was quantified using Jess automated western blot system using 25 protein normalization capillary cartridges following manufacturer's protocol (Bio-techne, Minneapolis, MN, USA). The proteins were detected, normalized and fold-change compared to the untreated group using Compass for Simple Western software (Bio-techne, Minneapolis, MN, USA).

2.14. Liver antioxidant tests

Antioxidant level of the liver was quantified using FRAP assay and glutathione (GSH) assay kits (Sigma, St. Louis, MO, USA) following method reported by Mohamad et al. (2015).

2.15. Serum cytokines quantification

Serum cytokines (IL-1α, IL-1β, IL-6, MIP-1α, MCP-1, TNF-α) was quantified using mouse XL cytokine performance assay kit using Luminex xMAP technology (R&D Systems, Minneapolis, MN, USA) following manufacturer protocol. The results were presented as the mean of fold change comparing the Treatment or Normal group to the Untreated group.

2.16. Liver iNOS and NF-kB expression

Total RNA was extracted from liver using RNeasy Mini Kit (Qiagen, Hilden, Germany) and 1st strand cDNA was synthesised from 1 μg of total RNA using NEXscript cDN synthesis kit (NEX Diagnostics, Gyeonggi-do, Korea) following manufacturer's protocols. Primers targeting mRNA for NF-kB, iNOS and β-actin were listed below:

nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB):
forward 5′-CATTCTGACCTTGCCTATCT-3′
reverse 5′-CTGCTGTTCTGTCCATTCT-3′;

inducible nitric oxide synthase (iNOS):
forward 5′-GCACCGAGATTGGAGTTC-3′
reverse 5′-GAGCACAGCCACATTGAT-3′;

β-actin:
forward 5′-TTCCAGCCTTCCTTCTTG-3′
reverse 5′-GGAGCCAGAGCAGTAATC-3′

Quantitative PCR (qPCR) was performed using NEXpro qPCR Evagreen master mix (NEX diagnostics, Korea) using SLAN-96P Real-Time PCR system (Sansure, Changsha, China) following steps: g steps: 95 °C for 2 mins, 40 cycles of 95 °C for 10s, 60 °C for 45 s. Expression level of the NF-kB and iNOS were normalized by β-actin and the fold change in the expression of each target genes was calculated by ΔΔCT method against the Untreated group.

2.17. Liver metabolomics analysis

Fresh liver samples were split into two, one for the extraction of polar metabolites, while another for the extraction of non-polar metabolites. About 100 mg liver sample was mixed with 5 mL of methanol and 5 mL of water for polar extraction, while non-polar extraction was conducted via the addition of 10 mL of chloroform and some sodium sulphate to remove moisture. All samples were kept dark in room temperature for overnight extraction. Followings, all samples were applied with 5000 rpm centrifugation for 10 mins, and the supernatants were recovered and dried with nitrogen gas flow. Dried tissue extracts were kept at −80 °C until further analysis. Prior to the LC-MS/MS analysis, liver extracts were re-constituted in 500 μL MeOH, and profiled as aforementioned in section 2.6.

2.18. Chemometric

The acquired data were pre-processed using Thermo Scientific Compound Discoverer 3.3 software (Thermo Fisher Scientific, Waltham, MA, USA) with the default metabolomics settings for background subtraction, retention time alignment, features detection, gaps filling, quality control (QC) correction and putative identification. Following, these data were exported in CSV format and merged within groups, prior to be analyzed via Metaboanalyst 6.0 (Pang et al., 2022; Pang et al., 2024) to determine the clustering of the control and Moringa treated groups as described in Ling et al. (2020). The identification of compounds was primarily based on the matching of MS/MS data against mzCloud and manual interpretation of MS/MS fragments according to Murphy (2014).

2.19. Statistical analysis

Data were displayed as means ± standard deviations (SD) and statistical significance for each group were analyzed using SPSS 16.0 (IBM, Armonk, NY, USA) by one-way analysis of variance (ANOVA) and Duncan's multiple range test. P values <0.05 were considered statistically significant.

3. Results & discussion

3.1. Sample identification

BLASTn results (Table 1) indicated that the Moringa leaf sample used in this study was indeed *M. oleifera*, with the highest score corresponding to accession number PQ000110.1, which refers to the sequence of *M. oleifera* isolate Mo32_Pond 5.8S ribosomal RNA gene and internal transcribed spacer 2, partial sequence.

Table 2
Correlation between the antioxidant assays with total phenolic and flavonoid contents.

Correlation (r^2)	DPPH	FRAP
TPC	0.93	0.83
TFC	0.90	0.79

3.2. Antioxidation activities, TPC, and TFC

The aqueous ethanolic extract of Moringa leaf were analyzed for antioxidation potential via FRAP assay and DPPH free radical scavenging activity. In present study, the aqueous ethanolic extract possess lower antioxidation activities in both DPPH and FRAP assays. The IC_{50} of the DPPH free radical scavenging activity is not achievable with the tested concentrations, while the antioxidation activity as per FRAP was determined at 13.41 mg GAE/g DW. In DPPH assay, the aqueous ethanolic extract only achieved 13.96% of inhibition at the highest tested concentration (0.5 mg/mL). On the other hand, TPC of the Moringa extract was determined at 24.73 mg GAE/g DW, whereas TFC was determined at 2.95 mg CE/g DW.

The correlation coefficients (r^2) between the antioxidant assays (DPPH and FRAP) with TPC and TFC of Moringa extract are presented in Table 2. The r^2 values ranged from 0.79 to 0.93, indicating strong positive correlations between antioxidant activities and bioactive compound contents. This is consistent with findings in other medicinal aromatic plants like *Nepeta leucophylla*, where high r^2 have been observed between TPC/TFC and the results of DPPH, FRAP, and TAC assays, confirming that polyphenolic constituents are the primary contributors to a plant's total antioxidant capacity (Sharma et al., 2021; Sharma & Cannoo, 2016; Sharma & Cannoo, 2017). Similar phenomena have been reported previously, where strong correlations were observed between the antioxidant capacity of various plant extracts and their polyphenolic content (Jacobo-Velázquez & Cisneros-Zevallos, 2009; Muflihah et al., 2021).

The stronger correlation observed with the DPPH assay suggests that phenolic and flavonoid compounds are the primary contributors to free radical scavenging activity, likely owing to their hydrogen- or electron-donating capacities. This observation is consistent with the underlying mechanism of the DPPH assay, which predominantly assesses radical quenching ability. In contrast, the comparatively lower correlation with FRAP may reflect the contribution of other bioactive reducing agents, such as carotenoids, terpenoids, tannins, alkaloids, and anthocyanins which exhibit varying degrees of antioxidant activity but are not quantified by TPC or TFC measurements (Gutiérrez-del-Río et al., 2021; Muflihah et al., 2021; Ngo et al., 2016; Terao, 2023). While DPPH evaluates the capacity to neutralize stable free radicals, FRAP measures reducing power under acidic conditions, potentially limiting its sensitivity toward certain phenolic subclasses. Consequently, some phenolic compounds may function more effectively as radical scavengers than as reductants, thereby explaining the weaker association with FRAP. These findings indicate that DPPH may be a more sensitive indicator of phenolic- and flavonoid-driven antioxidant activity in the studied samples; nevertheless, the application of multiple antioxidant assays remains essential to comprehensively evaluate overall antioxidant potential.

Generally, phenolic and flavonoid compounds are prone to be extracted with organic solvents, or more specifically semi-polar solvents, such as ethyl acetate. While Soxhlet extraction method with methanol often yield higher polyphenolic content (Sharma & Cannoo, 2016; Sharma & Cannoo, 2017), aqueous-ethanolic maceration is widely adopted for ensuring the solubility of polar compounds for consumption. As demonstrated by Mohammed et al. (2022), higher flavonoid contents were observed in extraction with ethanol as compared to methanol. In another study on Moringa leaf tissue extracts, the saline extracts demonstrated weak and undefinable antioxidation activities in conjunction with lower flavonoid contents as compared to ethanol extracts (Santos et al., 2012). This may be attributed to the prevalence of glycosylated phenolic compounds in Moringa leaves, which exhibit improved solubility in hydroalcoholic solvents, greater stability, and reduced toxicity compared with aglycones (Xie et al., 2022). The use of 80% ethanol in water therefore represents a practical compromise between extraction efficiency and safety, supporting its application in functional food development (Plaskova & Mlcek, 2023).

3.3. Metabolites profile

In present study, LC-q-Orbitrap-MS/MS was employed to profile the aqueous ethanolic extract of Moringa, and the acquired tandem mass spectrometry data has led to the identification of a total of 80 metabolites (57 in positive mode and 23 in negative mode) (Supplementary S1-S2). Among the detected metabolites, flavonoids and amino acids were

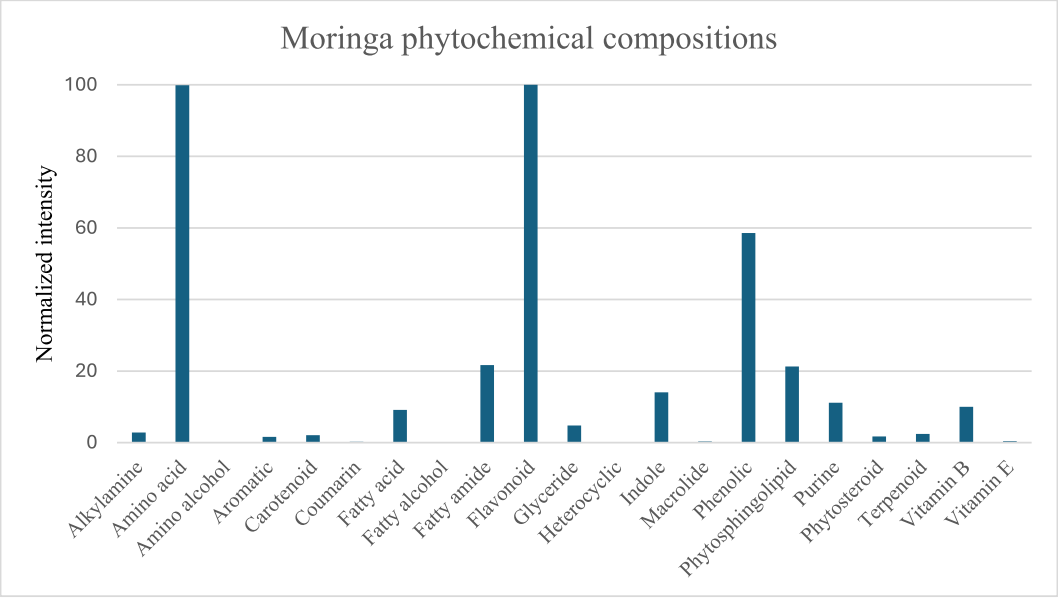


Fig. 1. Normalized phytochemical compositions of aqueous ethanolic extract of Moringa.

Table 3
Bioactivities of the flavonoids detected in the aqueous ethanolic extract of Moringa.

Compound	Flavonoids	Bioactivities	References
1	3″,4”-Diacetylafezelin	AC	(Matthes et al., 1980; Xu et al., 2006)
2	3-Methoxyluteolin	Anti-parasites	(Gallo et al., 2008)
3	6”-*O*-Acetylisoquercitrin	AO	(Kim & Jang, 2011)
4	6”-*O*-(3-Hydroxy-3-methylglutaroyl)hyperin		
5	8-*C*-Galactosylluteolin		
6	Cacticin	AD, AO	(Kim et al., 2011)
7	Isoquercetin	AC, AD, AI, AO, Cardioprotective, Neuroprotective	(da Silva et al., 2022; Jayachandran et al., 2018; Jayachandran et al., 2019; Yang et al., 2021; Zhang et al., 2011)
8	Isorhamnetin	AC, AH, AI, AM, AO, AV, Cardioprotective, Neuroprotective, Hormonal regulation	(Gong et al., 2020; Ishola et al., 2019; Khaled, 2020; Li et al., 2021)
9	Kaempferol	Hepatoprotective, AC, AD, AI, AM, AO	(Periferakis et al., 2022; Qattan et al., 2022; Ren et al., 2019; Tu et al., 2007; Yang et al., 2022)
10	Kaempferol 3-(6″-malonylgalactoside)		
11	Quercetin	AC, AI, AM, AO, AV	(Aghababaei & Hadidi, 2023; Boots et al., 2008; Kicel & Wolbiś, 2012; Murakami et al., 2008)
12	Quercetin 3-*O*-malonylglucoside		
13	Trifolin	AC, AF, AO	(Kicel & Wolbiś, 2012; Kim et al., 2016; Li et al., 2005)
14	Vicenin-2	AC, AI, AM, AO, Hepatoproctetive, Wound healing	(Duan et al., 2019; Marrassini et al., 2011; Tan et al., 2019; Yang et al., 2018; Zhang et al., 2021)
15	Vitexin	AC, AI, AO, Neuroprotective	(Babaei et al., 2020; He et al., 2016)

AC: Anti-cancer.
AD: Anti-diabetic.
AF: Anti-fungal.
AH: Anti-hyperuricemia.
AI: Anti-inflammation.
AM: Anti-microbial.
AO: Anti-oxidation.
AV: Anti-viral.

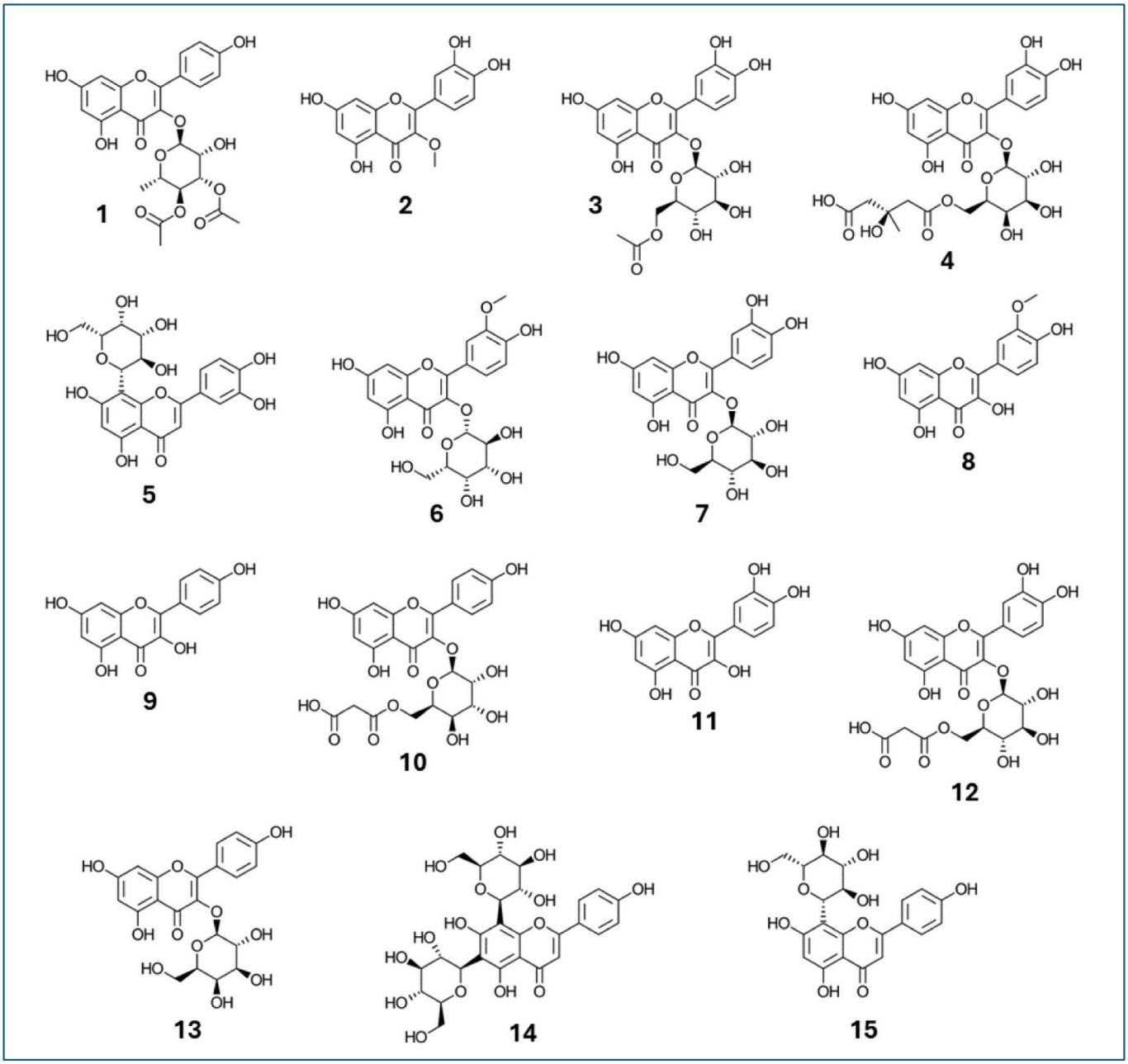
identified as the major composition of the extract, followed by phenolics, phytosphingolipids and fatty amides (Fig. 1). The finding is aligned with the antioxidant and TPC profile, on which higher content of polar metabolites was detected in the aqueous ethanolic extract. Interestingly, there are various bioactive metabolites were detected in this aqueous ethanolic extract of Moringa leaf, and the reported bioactivities include antioxidation, anticancer, antidiabetic, antimicrobial, antiviral, neuroprotective, cardioprotective, hepatoprotective, hormonal regulation, and etc. (Abdellatif et al., 2021; Aghababaei & Hadidi, 2023; Li et al., 2021; Razzaq et al., 2023; Takemura et al., 2021).

Among the detected metabolites, flavonoids, phenolics, carotenoids, indoles, terpenoids and vitamins, are the common bioactive classes which are well known possessing antioxidation properties. 2-Hydroxycinnamaldehyde, which also known as *o*-coumaraldehyde, is a phenolic compound which was reported with significant antioxidative and anti-hemolysis activities (Jiang et al., 2014). In addition, cinnamaldehydes were reported to possess cancer inhibition activities via hormonal regulation. In an in vivo study reported by Boggiti & Penchalaneni (2021), progesterone was found upregulated in the female Wistar rats which administered cinnamaldehyde, thus supported the claims in countering ovarian cancers (Banerjee & Banerjee, 2023). Pulegone, a monoterpene ketone that was reported with various bioactivities, was identified in the aqueous ethanolic extract of Moringa. Literature revealed that the pulegone possess anti-inflammatory, antihyperalgesic, antimicrobial, antihypertensive, and vasoprotective activities (Flamini et al., 1999; Hilfiger et al., 2021; Razzaq et al., 2023; Roy et al., 2018).

On the other hand, the detection of nicotinamide and α-tocospiro A in the extract demonstrated the potential of the extract to be employed as health supplements due to their wide scales of health enhancement mechanisms. According to Maiese (2021), nicotinamide is identified as cytoprotectant against acute and chronic neurodegenerative disorders, which is essential to prevent and reverses neuronal and vascular cell injury. In addition, the antioxidation characteristic of nicotinamide was found useful in skin aging and pigmentation control, while preventing skin cancer (Allen et al., 2023; Hakozaki et al., 2002; Park, Byun et al., 2022; Scatozza et al., 2020). Besides, α-tocospiro A, which is commonly known as vitamin E derivative or tocopheroid, was firstly isolated from *Ficus microcarpa* and reported possessing anti-inflammatory, antiproliferative, and antituberculosis activities (Chen et al., 2010; Chiang & Kuo, 2003; Park, Lee et al., 2022).

Overall, there are 15 different flavonoids were identified from this extract (Table 3) and their structures were illustrated in Fig. 2, including cacticin, isoquercetin, isorhamnetin, kaempferol, quercetin, trifolin, vicenin-2 and vitexin. Present study demonstrated higher variety of flavonoids as compared to the optimized extraction protocol which employs mixture of choline chloride and glycerin as extraction solvent, thus supported the feasibility of aqueous ethanolic extraction practice in present study (Wei et al., 2023). Besides, present findings are found aligned with earlier studies, where kaempferol and quercetin were reported to be the main flavonoids in Moringa extract, followed by the vitexin and isorhamnetin (Coppin et al., 2013; Lin, Zhang & Chen, 2018; Makita et al., 2016; Wei et al., 2023). Among the detected flavonoids, most of them were reported possessing wide spectrums of bioactivities (Table 3). On top of the common bioactivities, kaempferol was found stimulating the hormonal homeostasis. As highlighted by da-Silva et al. (2007), kaempferol stimulated the expression of *Dio2* gene and leads to the elevated D2 activity and T3 thyroid hormone synthesis production in intact cells, which increase energy expenditure and countering diet-induced obesity. Furthermore, kaempferol was found triggered various signalling pathways, such as insulin, estrogen, prolactin, p53, and etc., against bone disorders (Guo et al., 2012; Tang et al., 2022). On the other hand, elevation of prolactin was observed post 10 days of quercetin administration along with the increased yield of milk production in an in vivo study on mice Lin et al., 2018.

In the present study, kaempferol, luteolin, quercetin, and vitexin were selected for further quantitative analysis following the qualitative analysis via LC-MS/MS. These flavonoids were selected mainly due to



1: 3",4"-diacetylafezelin; **2**: 3-Methoxyluteolin; **3**: 6"-*O*-acetylisoquercitrin; **4**: 6"-*O*-(3-hydroxy-3-methylglutaroyl)hyperin; **5**: 8-*C*-galactosylluteolin; **6**: cacticin; **7**: isoquercetin; **8**: isorhamnetin; **9**: kaempferol; **10**: kaempferol 3-(6"-malonylgalactoside); **11**: quercetin; **12**: quercetin 3-*O*-malonylglucoside; **13**: trifolin; **14**: vicenin-2; **15**: vitexin

Fig. 2. Chemical structures of the detected flavonoids as listed in Table 3.

their reported bioactivities. To improve the signal resolution, chemical standards were spiked into the extract and quantified via a standard addition curve. In the present study, vitexin showed the highest content among the selected flavonoids, followed by kaempferol and luteolin (Supplementary S3). The coefficient of determination (R^2) for all four selected flavonoids was recorded above 0.995, indicating high reliability of the developed HPLC protocols. Nonetheless, baseline separation is unattainable with our established HPLC methods owing to the complexity of crude extracts, and coelution of flavonoids is unavoidable due to their structural resemblance. For better quantification of flavonoids, the application of multiple reaction monitoring (MRM) with an LC-MS/MS system is recommended.

Literature reveals that kaempferol and quercetin were anticipated to be the predominant flavonoids, contributing to higher levels compared to vitexin and luteolin (Coppin et al., 2013; Lin, Zhang & Chen, 2018; Makita et al., 2016; Wei et al., 2023). Nevertheless, quercetin was recorded at the lowest level among the selected flavonoids in the present study. This phenomenon might be attributed to the initial extraction process, which employs an aqueous-ethanolic extraction approach used in this study. Liau et al. (2017) revealed that aqueous-ethanolic extraction has a higher extraction efficiency compared to 100% ethanol, resulting in a considerable increase in the yield of polar flavonoids, such as glycosides. Among the selected flavonoids, vitexin is the only glycoside, hence exhibiting greater affinity for the present

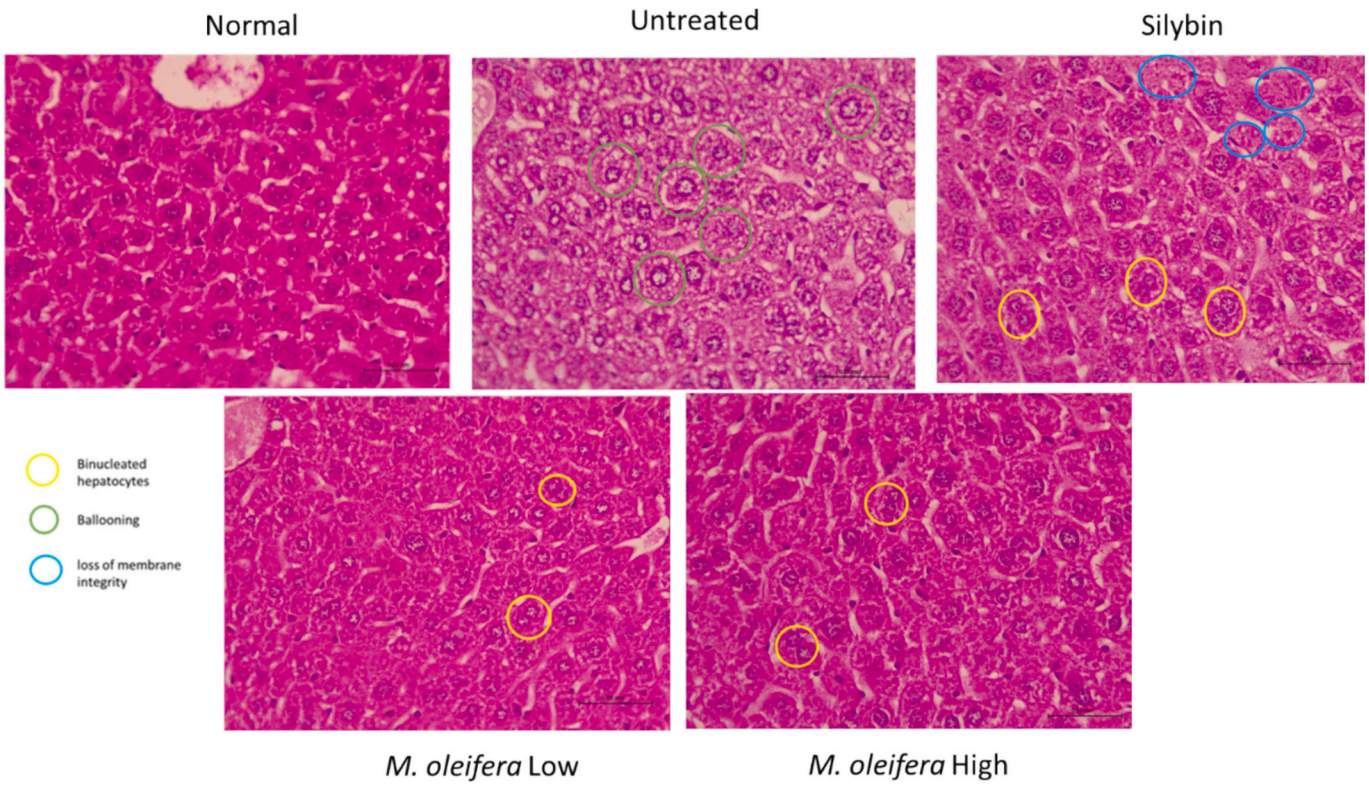


Fig. 3. Liver Hematoxylin and Eosin (H&E) stained histopathology analysis. Ballooning was observed in the liver of untreated mice. Liver from Silybin and Moringa treated mice show similar histological appearance as normal liver with higher incidence of binuclear hepatocyte. Normal: normal control; Untreated: APAP-challenged mice fed with distilled water; Silybin: Silybin 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa Low: *M oleifera* 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa High: *M. oleifera* 200 mg/kg body weight (BW) treated APAP-challenged mice.

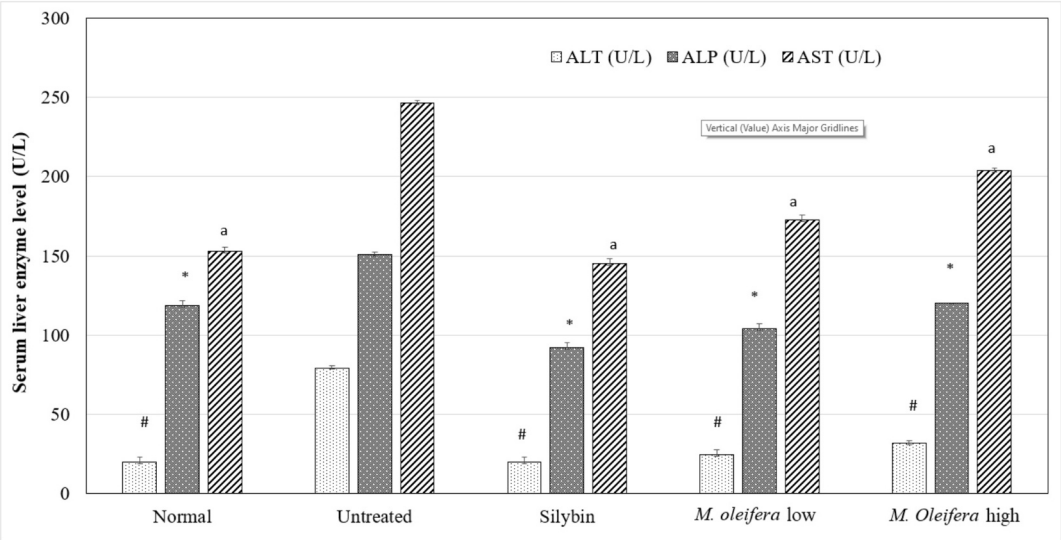


Fig. 4. Serum ALT, ALP and AST markers. Ballooning was observed in the liver of untreated mice. Liver from Silybin and Moringa treated mice show similar histological appearance as normal liver with higher incidence of binuclear hepatocyte. Normal: normal control; Untreated: APAP-challenged mice fed with distilled water; Silybin: Silybin 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa Low: *M. oleifera* 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa High: *M. oleifera* 200 mg/kg body weight (BW) treated APAP-challenged mice. Statistical significance between the groups ($^{\#,*,a}p < 0.05$) compared with untreated group.

extraction process.

1: 3″,4″-diacetylafezelin; **2**: 3-Methoxyluteolin; **3**: 6“-*O*-acetylisoquercitrin; **4**: 6”-*O*-(3-hydroxy-3-methylglutaroyl)hyperin; **5**: 8-*C*-galactosylluteolin; **6**: Cacticin; **7**: Isoquercetin; **8**: Isorhamnetin; **9**: Kaempferol; **10**: Kaempferol 3-(6″-malonylgalactoside); **11**: Quercetin; **12**: Quercetin 3-*O*-malonylglucoside; **13**: Trifolin; **14**: Vicenin-2; **15**: Vitexin

3.4. *In vivo study*

All mice survived until 30 days of the treatment. Ballooning with larger cell-cell gap were observed in the hepatocytes of the liver

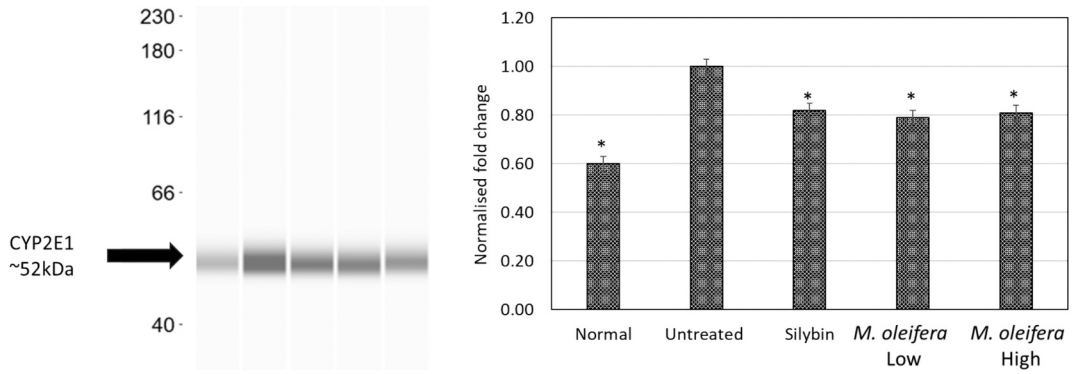


Fig. 5. Liver protein expression of cytochrome P450 2E1 (CYP450 2E1). Silybin and *M. oleifera* treated mice show lower expression level of CYP450 2E1 than untreated mice in the liver. Normal: normal control; Untreated: APAP-challenged mice fed with distilled water; Silybin: Silybin 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa Low: *M. oleifera* 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa High: *M. oleifera* 200 mg/kg body weight (BW) treated APAP-challenged mice. Statistical significance between the groups (*$p < 0.05$) compared with untreated group.

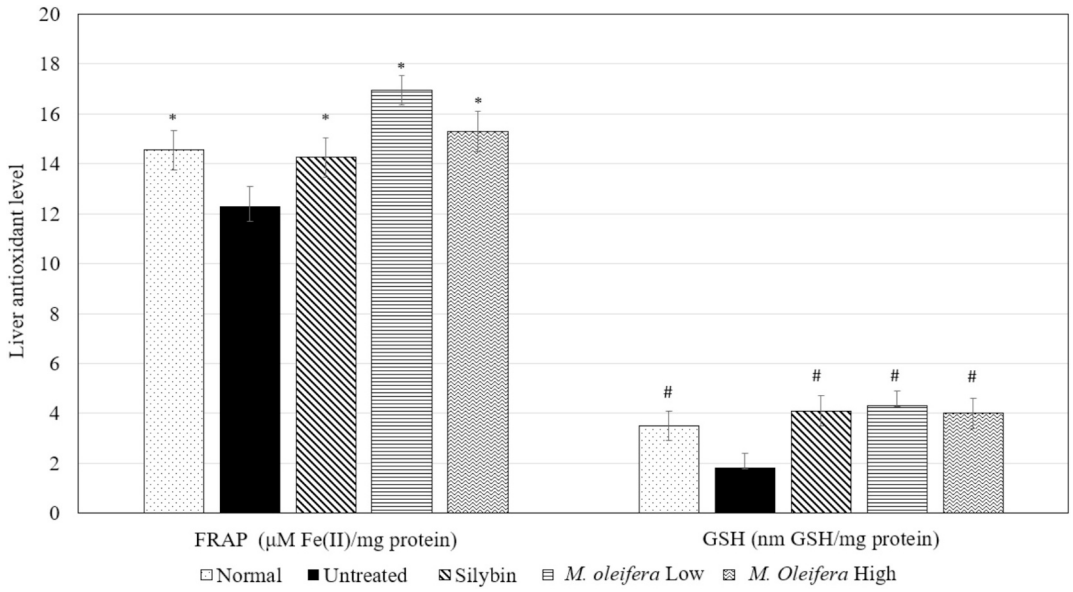


Fig. 6. Liver antioxidant level measured by FRAP assay and GSH. Silybin and *M. oleifera* treated mice show higher level of FRAP total antioxidant and GSH level than untreated mice in the liver. Normal: normal control; Untreated: APAP-challenged mice fed with distilled water; Silybin: Silybin 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa Low: *M. oleifera* 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa High: *M. oleifera* 200 mg/kg body weight (BW) treated APAP-challenged mice. Statistical significance between the groups ($^{*,\#}p < 0.05$) compared with untreated group.

histology from the untreated mice (Fig. 3). Liver from Silybin and Moringa treated mice appeared normal with some incidence of binucleated hepatocytes. Previous study on the hepatoprotective effect of gallic acid against APAP-induced liver damage also reported with binucleated hepatocytes in the liver histology study (Rasool et al., 2010).

Liver enzyme markers such as ALT, ALP and AST were commonly located in the cytosol of hepatocytes. Thus, leakage of these enzyme markers in the bloodstream was important diagnostic markers to evaluate level of hepatic injury (Sharifudin et al., 2013). APAP increased the serum ALT, ALP and AST markers comparing to the normal healthy mice (Fig. 4). Both Silybin and Moringa extracts were able to significantly reduced the all-serum liver enzyme markers comparing to the untreated group. Restoring of hepatocytes structure indicated by the binuclear hepatocytes observed in the histological study supported the liver enzyme markers results where repaired hepatocytes has less leakage of those intracellular enzyme into the serum.

Cytochrome P450 2E1 (CYP450 2E1) was known to convert APAP into highly reactive intermediate metabolite *N*-acetyl-p-benzoquinone imine (NAPQI), which can be rapidly detoxified by glutathione (GSH). Overexpression of CYP450 2E1 associated with reduction of total antioxidant level particularly depletion of GSH were reported in the APAP-induced liver damage (Mohamad et al., 2015; Yan et al., 2018). Downregulation of this enzyme was observed in the mice that recovered from the APAP-induced hepatotoxicity (Mohamad et al., 2015). In this study, normal mice was recorded with lowest CYP2E1 expression (Fig. 5) and high level of FRAP antioxidant and GSH level (Fig. 6). On the other hand, untreated mice have highest expression of CYP450 2E1 (Fig. 5) and lowest FRAP antioxidant and GSH levels (Fig. 6). Silybin and Moringa treatments were observed with lower expression of CYP450 2E1 than the untreated group (Fig. 5). Based on the current results, Moringa extract was able to restore the antioxidant level, which prevent the APAP-induced liver damage indicated by both histology and serum liver enzyme profile level.

Overdose of APAP was associated with acute inflammatory response due to damage of liver cells activated overexpression of proinflammatory cytokines such as TNF-alpha, IL-1beta, IL-6 and chemokines such as MCP-1, MIP-2 and IL-8. Although the role of inflammation response to the outcome of drug-induced liver damage was debatable, proinflammation response associated with high serum liver enzyme

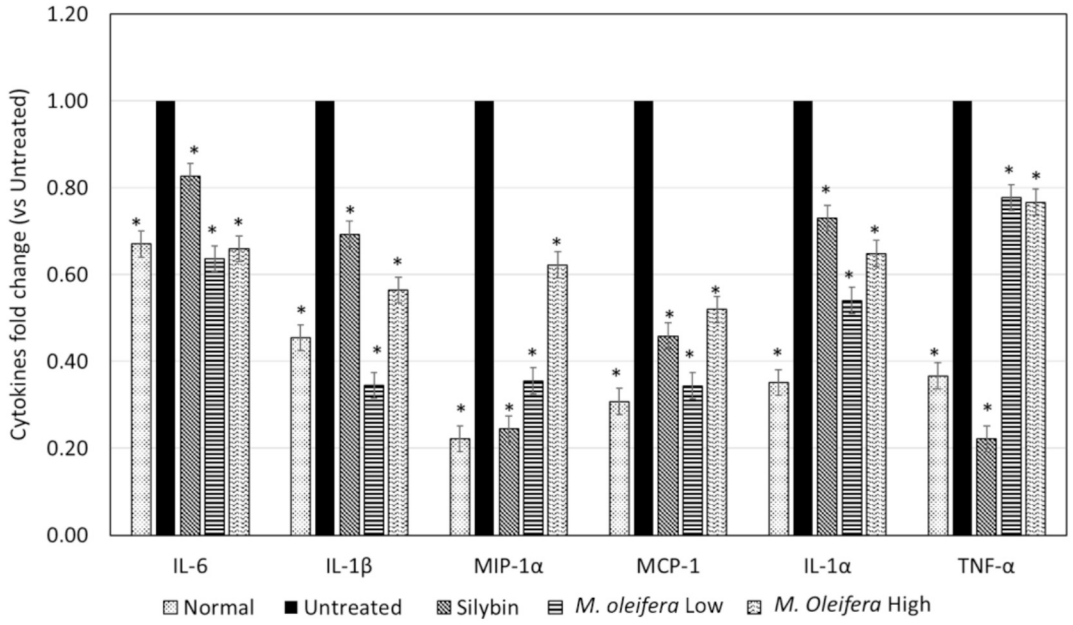


Fig. 7. Serum proinflammatory cytokines (IL-6, IL-1α, IL-1β, MIP-1, MIP-1α, TNF-α). Silybin and Moringa treated mice show lower serum proinflammatory cytokines level than untreated mice. Normal: normal control; Untreated: APAP-challenged mice fed with distilled water; Silybin: Silybin 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa Low: *M. oleifera* 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa High: *M. oleifera* 200 mg/kg body weight (BW) treated APAP-challenged mice. Statistical significance between the groups (*$p < 0.05$) compared with untreated group.

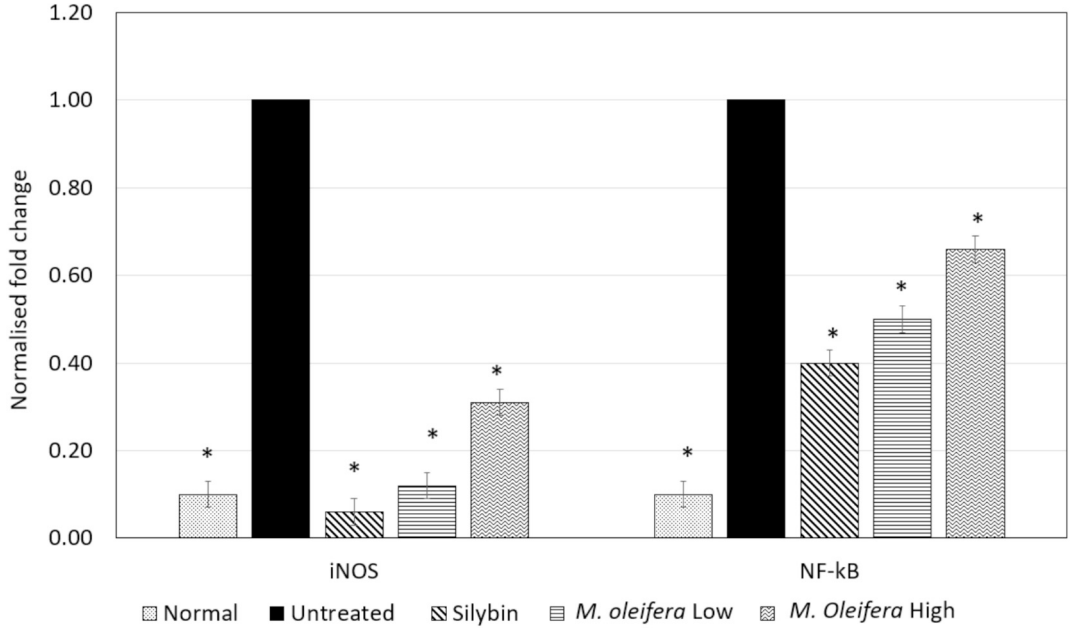


Fig. 8. Liver mRNA expression of proinflammatory genes (iNOS and NF-kB). Silybin and Moringa treated mice show lower expression level of iNOS and NF-kB genes than untreated mice in the liver. Normal: normal control; Untreated: APAP-challenged mice fed with distilled water; Silybin: Silybin 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa Low: *M. oleifera* 50 mg/kg body weight (BW) treated APAP-challenged mice; Moringa High: *M. oleifera* 200 mg/kg body weight (BW) treated APAP-challenged mice. Statistical significance between the groups (*$p < 0.05$) compared with untreated group.

profile was accepted as positive correlation indicating liver damage (Yan et al., 2018). In this study, untreated mice were recorded with the highest serum proinflammatory cytokines level (Fig. 7) and serum liver enzyme profile (Fig. 4) indicating overdose of APAP damaged the liver. Blockage of IL-1 and TNF-α exhibit reduced APAP-induced liver damage in the previous study (Xu & Wang, 2023). Preliminary study by Aly et al. (2020) has shown reported 2 months treatment of Moringa extract was able to reduce TNF-α and TGF-β level associated with reduction of serum liver enzyme profiles. Similarly, Silybin and Moringa extract were able to reduce all the evaluated proinflammatory cytokines level in the serum indicating that both extracts were able to suppress the APAP-induced inflammation.

Proinflammatory cytokines including IL-1β and TNF-α were able to activate the NF-kB pathway. NF-kB is the transcription factor that the downstream inflammation related genes including iNOS that promote accumulation of Nitric Oxide (NO) (Guo et al., 2024). Oral administration of Silybin and Moringa significantly decreased the iNOS and NF-κB expression levels in the liver compared with the APAP untreated group.

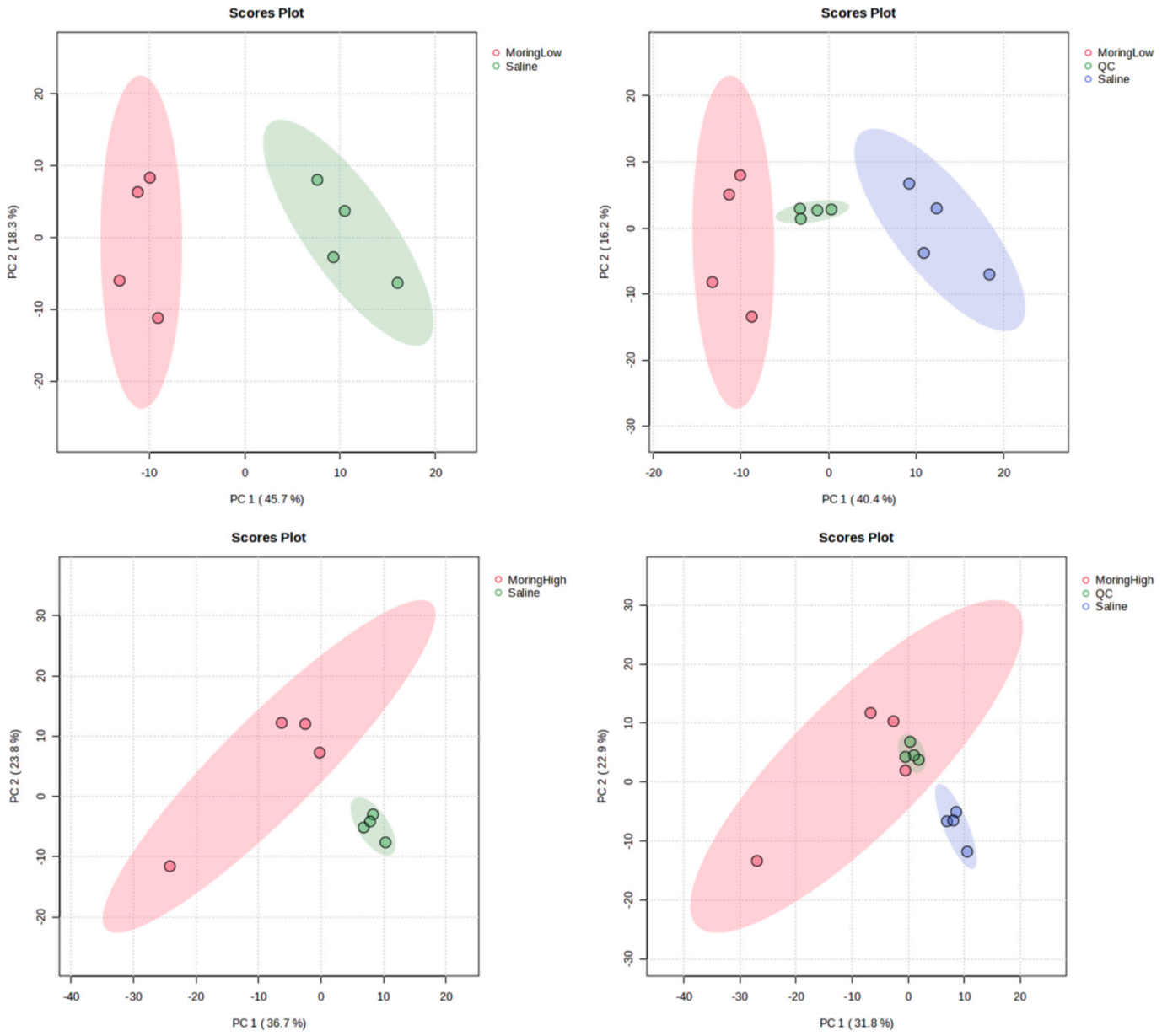


Fig. 9. PCA plot of liver metabolome for Moringa low and high compared to untreated mice.

Downregulation of iNOS was greatest than the NF-kB in both Silybin and Moringa treated group (Fig. 8).

3.5. Liver metabolomics

PCAs revealed the metabolome extracted from the livers demonstrated distinct between Moringa (low and high) treatments and control group (Fig. 9) profiled under positive and negative ionization mode. Briefly, there is a total of 231 and 118 features were significantly (p-value <0.05) perturbed metabolites post-exposure to low and high dose Moringa treatments, respectively. Among these, a total of 160 and 92 features were identified in low and high Moringa treatment groups, respectively (Supplementary S4-S5). There is a contrast different between low and high Moringa treatment groups in terms of phospholipids and sphingolipids perturbation, on which there are 53 phospholipids and 7 sphingolipids were perturbed in low Moringa treatment as compared to 13 phospholipids and 3 sphingolipids These lipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingomyelin (SM), and ceramide (Cer). The perturbation of lipids in liver cells are often associated with lipotoxicity and programmed cell death, on which this phenomenon is aligned with the uptake of acetaminophen (Alkhouri et al., 2009; Suciu et al., 2015). According to Chen et al. (2009), acetaminophen-induced hepatotoxicity leads to the oxidative stress and caused the inhibition of fatty acid β-oxidation. However, downregulation of acylcarnitine in both treatment groups were noticed, which indicating that the fatty acid β-oxidation in liver cells are recovering following the intake of Moringa extracts. Generally, acylcarnitines are esters of L-carnitine and fatty acids, which acting as the carriers to transport fatty acids into mitochondria for subsequent β-oxidation for cellular energy generation (Li et al., 2019). In the event that the fatty acids β-oxidation is inhibited, accumulation of acylcarnitines should be observed. While comparing the lipids and acylcarnitines perturbation against untreated mice, low Moringa treatment was observed demonstrating better recovery due to higher metabolites changes as compared to high Moringa treatment in response to APAP-induced stress assay. While an increase in apoptosis-inducing ceramides was noted in both Moringa treatment groups, the low Moringa treatment exhibited a more

pronounced elevation. The activation of apoptosis may initiate apoptosis-induced compensatory proliferation (AiP), which facilitates wound healing and tissue regeneration. (Diwanji & Bergmann, 2018; Ichim & Tait, 2016).

On the other hand, upregulation of multiple prostaglandins in low Moringa treatment group is associated with inflammatory response of the liver cells, and this perturbation is not observed in high Moringa treatment group. Briefly, prostaglandins promote inflammation in response to infection and injury, which is essential in reversing adverse factors and restoring tissue structure and physiological functions (Peltekian et al., 1996; Ricciotti & FitzGerald, 2011). According to Shimada et al. (2020), elevation of prostaglandin E_2 metabolites in low Moringa treatment group is associated with tissue protection and regeneration via the induction of hepatocytes proliferation and lower the production of inflammatory cytokines. Along with the prostaglandin E2, upregulation of prostaglandin E1 in liver tissue was reported promoting hepatocytes proliferation via the inhibition of reactive oxygen species (Liu & Fan, 2000).

Among the perturbed steroids, cortisol was observed upregulated in the high Moringa treatment group, but not in low Moringa treatment group. Cortisol is known as stress hormone, and the elevation of cortisol often associated to physical and/or emotional stress (Burke et al., 2005; Dedovic et al., 2009; Oswald et al., 2006). Thus, upregulation of cortisol indicates that the animal subjects were in higher stress following high Moringa treatment as compared to their low Moringa treatment counterpart.

4. Conclusion

This study comprehensively characterized the phytochemical composition, antioxidant capacity, and hepatoprotective potential of *M. oleifera* aqueous ethanolic extract. Despite moderate in vitro antioxidant activity, the aqueous ethanolic extract retained a diverse array of bioactive metabolites, such as flavonoids, phenolics, amino acids, phytosphingolipids, fatty amides, carotenoids, terpenoids, indoles, and vitamins, which supporting its wide-ranging pharmacological effects. Notably, 15 distinct flavonoids were identified, demonstrating the efficacy of aqueous ethanolic extraction in preserving polar bioactive compounds suitable for nutraceutical applications. In vivo treatment with 50 mg/kg body weight of the extract significantly ameliorated APAP-induced hepatotoxicity in mice over 30 days, primarily through antioxidant and anti-inflammatory mechanisms. Metabolomic analysis further revealed that the extract restored lipid metabolism and upregulated prostaglandin pathways, promoting hepatocyte recovery by inhibiting reactive oxygen species and proinflammatory mediators. Collectively, these findings provide strong evidence for the therapeutic potential of Moringa aqueous ethanolic extract in managing drug-induced liver injury and reinforce its value as a functional ingredient in health and nutritional formulations.

CRediT authorship contribution statement

Yoong Soon Yong: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Michelle Siao:** Writing – review & editing, Validation, Resources, Funding acquisition, Data curation, Conceptualization. **Kam Heng Ng:** Writing – review & editing, Validation, Resources, Funding acquisition, Data curation, Conceptualization. **Boon Ful Ng:** Writing – original draft, Visualization, Project administration, Methodology, Investigation, Data curation. **Jen Kit Tan:** Writing – review & editing, Visualization, Software, Funding acquisition, Data curation, Conceptualization. **Nurul Fatimah Mohd Nasir:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Data curation. **Wen Siang Tan:** Writing – review & editing, Validation, Supervision, Resources, Investigation, Data curation. **Noorjahan Banu Alitheen:** Writing – review & editing, Visualization, Validation, Resources, Investigation, Formal analysis. **Swee Keong Yeap:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Formal analysis, Conceptualization. **Chean Yeah Yong:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Formal analysis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2026.107232.

Data availability

Data will be made available on request.

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