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In vivo toxicity study in Sprague–Dawley rats receiving different doses of *Moringa oleifera* extract

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Abstract

Background: Protein deficiency poses a significant challenge during the growth and development of children. *Moringa oleifera* Lam (MO) leaves, renowned for their high protein content, present a potential solution to address amino acid imbalances in protein-deficient conditions.

Aim: This study aimed to evaluate the toxicity of MO leaf extract in Sprague-Dawley Rats.

Methods: Protein extraction from *Moringa oleifera* (MO) leaves was performed using ultrasonic-assisted extraction (UAE) with ethanol. The ethanol leaf extract of MO (EEMO) was then characterized for protein content, amino acids, minerals, phytic acid, and phytochemicals. Antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) test, while cytotoxicity was evaluated using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide) assay on HepG2 and Madin-Darby canine kidney (MDCK) cells. EEMO was tested for acute toxicity in 60 healthy male and female Sprague–Dawley rats. The rats were divided into five groups of five rats each, receiving single oral doses of 5, 50, 300, 2,000, and 5,000 mg/kg body weight (BW) of EEMO. Observations were conducted daily till day 14 before the necropsy of rats. Liver and kidney tissues were harvested and preserved in 10% formalin for histopathological analysis.

Results: The extraction process revealed a protein content of 45.5%, with phenylalanine being the predominant essential amino acid at 22.25 mg/g, and glutamic acid as the dominant non-essential amino acid at 60.03 mg/g. Potassium (1174.23 mg/100 g) and selenium (149 mg/100 g) were identified as the primary macro and micro minerals, respectively. The IC_{50} and CC_{50} values for antioxidant and cytotoxic activities in HepG2 and MDCK were found to be 41.04,182.66, and 121.04 ppm, respectively. Toxicity testing on experimental animals resulted in an LD_{50} value of 5,000 mg/kg BW for EEMO, indicating its relative safety upon oral administration.

Conclusion: MO extract produced by the UAE extraction method, containing high-quality food-grade protein, showed no cytotoxic effects on HepG2 and MDCK cells and exhibited no acute toxicity in rats.

Keywords: Antioxidant, Cytotoxicity, *Moringa oleifera*, Protein, Toxicity, Ultrasound assisted extraction.

Introduction

Protein plays a vital role in cellular maintenance and tissue building, a function that cannot be replaced by other nutrients. A deficiency in protein can significantly impact tissue development and cellular metabolism. In children, protein deficiency often manifests as underweight and stunting, with repercussions on the immune system, hepatic steatosis, muscle mass reduction, and cognitive disorders or delays in intellectual development (Bourke *et al*., 2019).

Various interventions have been explored to address protein deficiency, including the provision of food supplements (Bourke *et al*., 2019). Protein sources can be derived from both plant and animal origins. While animal proteins are advantageous due to their high protein content and good digestibility, they are often expensive and inaccessible for those living below the poverty line (Gidamis *et al*., 2003).

The *Moringa oleifera* Lam (MO) plant from Moringaceae family, known for its high protein content, thrives in various seasons and is drought-

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resistant, making it an excellent source of plant protein. Particularly, MO leaves are rich in fiber, vitamins, minerals, proteins, and other significant phytochemicals. Extensive research has shown that MO leaves contain protein levels ranging between 23% and 27% (Teixeira *et al*., 2014).

MO extracts, derived from leaves, seeds, and bark, are highly concentrated in antioxidants, anti-inflammatory agents, and other beneficial compounds with medicinal properties. These extracts are often included in supplements, skincare, and health and wellness products (Matic *et al*., 2018; Tshabalala *et al*., 2019; Granella *et al*., 2021). Methods such as cold pressing, solvent extraction, and supercritical fluid extraction (SFE) are utilized to obtain these extracts. Several studies have highlighted that the highest protein content was found in leaf flour, dried leaves, and fresh leaves (Gidamis *et al*., 2003; Peñalver *et al*., 2022).

Plant proteins, such as those from *Spirulina platensis*, *Camellia sinensis* leaves, and *Moringa oleifera* (MO) leaves, are generally cell-bound, necessitating effective extraction methods to release them (Garcia-Salas *et al*., 2010; Yucetepe *et al*., 2018; Menezes *et al*., 2019; Cheng *et al*., 2021). Traditional techniques, such as maceration, are often used for extracting bioactive or phytochemical compounds. However, these methods have several disadvantages, including the need for large quantities of solvents or reagents, daily solvent replacement, environmental concerns, prolonged extraction times (3–5 days), lower protein content, and reduced yields in the final extract (Alhakmani *et al*., 2013; Vongsak *et al*., 2013; Zeitoun *et al*., 2020).

Recently, novel extraction technologies like ultrasound-assisted extraction (UAE) have attracted attention for their reduced processing times, lower energy consumption, and higher extraction efficiency (Garcia-Salas *et al*., 2010; Cao *et al*., 2023). Fatima *et al*. reported a protein extraction yield of 39.12% from MO seeds using UAE, with an optimal extraction time of 20 minutes (Fatima *et al*., 2023). Similarly, Kingwascharapong *et al*. achieved optimal protein extraction from *Bombay locusts* (*Patanga succinta L*.) under UAE conditions with 60% amplitude and a 20-minutes extraction time (Kingwascharapong *et al*., 2021).

This study aims to characterize the compounds extracted from MO using the UAE method and to examine their acute toxicity in an animal model. These assessments are essential to determine the appropriate dosage of the extracts for potential use in humans, as required by the National Food and Drug Agency (BPOM, 2022).

Materials and Methods

Sample collection and species identification

MO leaves were collected from November 2021 to March 2022 from Kupang Regency in East Nusa Tenggara (NTT), Indonesia. To confirm the species of the MO leaves, samples were submitted to the Depokensis Herbarium (DEP) at the University of Indonesia's Biota collection room with certificate number 851/UN2.F3.II/PDP.02.00/2021 on December 1, 2021; and to the Bogoriense Herbarium with certificate number B-168/II.6.2/DI.05.07/6/2022 on June 7, 2022. Species identification was carried out using reference materials and comparisons with herbarium collections in the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia by Mr. Alexander Tianara, Botanical and Zoological Specimen Curator of Biota Collection Room, and Mr. Alex Sumadijaya, Plant Systematist at Herbarium Bogoriense, Research Center for Biosystematics and Evolution, from Bogoriense Herbarium, Directorate of Scientific Collection Management, National Research and Innovation Agency (BRIN). The voucher specimen of MO is maintained in the Directorate of Scientific Collection Management, BRIN-Indonesia.

Sample preparation

A total of 2.8 kg of fresh MO leaves underwent several preparation steps. Initially, the leaves were thoroughly washed and air dried. They were then dried for 24 hours in a 42°C oven, a temperature safe for preserving the protein content. The weight of the dried leaves was recorded. Subsequently, the dried leaves were finely ground into a powder using a knife mill to achieve a particle size of 100 mesh. The resultant MO powder was carefully stored in a sealed container to maintain its quality. All methods were conducted in accordance with the International Union for Conservation of Nature and Natural Resources (IUCN) Policy Statement on Research Involving Species at Risk of Extinction.

Ultrasonic assisted extraction

Ultrasonic-assisted extraction (UAE) of MO from dried leaves followed a modified procedure outlined by Dadi *et al*. (2019a). Fifty grams of MO leaf powder was mixed with 70% ethanol in a 1:10 (w/v) ratio. The extraction was performed using an ultrasonic bath (Ultrasonic H-D Ultra Selecta®) operating at 40 kHz, at 40°C, with an ultrasonic input power of 400 W. The mixture was then filtered through Whatman filter paper no. 1. The filtrate was concentrated using a rotary evaporator system (Buchi® R-215 Rotary Evaporator with B-491 Heating Bath, V700 Vacuum Pump, V850 Vacuum Controller and F-100 Chiller) under the following conditions: vapor temperature of 50°C, system pressure of 175 mbar, bath temperature of 50°C, and condenser temperature of 20°C. The concentrated solution was freeze-dried for 24 hours using a Hypercool® Cooling Trap HC3110 at −110°C and 1.3×10^{-1} Pa to obtain dry extracts, which were weighed and stored at 4°C in a light-protected container.

Different durations of ethanol incubation were applied in this study. To ensure the reliability of the method, the extraction process using ethanol for 60 minutes, which produced a high protein content, was repeated four times.

Characterization of ethanol extract of MO leaves (EEMO)

EEMO was analyzed for protein, proximate chemical, mineral, and amino acid content, as well as mycotoxin contamination, moisture content, ethanol content, total plate count, yeast mold, bacterial contamination (including *Escherichia coli, Enterobacteriaceae, Salmonella sp., Shigella sp.,* and *Clostridium*), and heavy metal contamination. These tests are necessary to meet the standard qualifications set by the National Food and Drug Agency of Indonesia Regulation Standard Number 32 of 2019 (BPOM RI, 2019). All tests were conducted by SNI ISO/IEC 17025:2017-certified laboratories, PT. Saraswanti Indo Genetech, Jakarta, Indonesia, accredited by the National Accreditation Committee (KAN) with number LP-184-IDN.

Phytic acid content determination

The phytic acid content was determined using the method described by Davies *et al*. (1979). First, 1 g of the sample was suspended in 50 ml of 0.5 M HNO_3. This suspension was stirred with a magnetic stirrer for 2 hours at room temperature and then filtered to obtain the filtrate. The phytic acid content was analyzed by mixing 0.5 ml of the filtrate, 0.9 ml of 0.5 M HNO_3, and 1 ml of 0.3 mM $FeCl_3$ in a test tube. The test tube was closed and then soaked in boiling water for 20 minutes. After cooling down, 5 ml of amyl alcohol and 1 ml of 0.1 mM ammonium thiocyanate were added, followed by centrifugation (Eppendorf® EP-5804) at 1.500 rpm for 10 minutes. Once two layers were formed, the absorbance of the amyl alcohol layer was measured using a spectrophotometer (Agilent® Technology Type Cary 60) at a wavelength of 465 nm, with an amyl alcohol blank measured 15 minutes after adding ammonium thiocyanate.

Phytochemical analysis

The phytochemical components of EEMO were identified using liquid chromatography mass spectrometry/mass spectrometry (LCMS-MS) in positive ion mode. The extract was dissolved in an appropriate solvent and injected in a volume of 1 µl of water for analysis. The electrospray ionization (ESI) mode was set to full scan m/z of 100–1,200. The mobile phase consisted of H_2O+0.1% formic acid and acetonitrile + 0.1% formic acid. Analysis was performed on an LCMS-MS Waters™ Acquity UPLC-1 Class and XEVO G2-XS QT of, ACQUITY UPLCTM BEH C18 column (1.7 µhm 2.1 × 50 mm), and XEVO G2-XS TOF mass spectroscopy.

Antioxidant activity assay

The possible antioxidant activities of EEMO were investigated using the DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) assay as described by Desmarchelier *et al*. (1997) and Rahman *et al*., (2015). Extracts at various concentrations (12.5–150 g/ml) were mixed with 2.4 ml of 0.1 mM DPPH. The solution mixture was vortexed and stored in the dark at room temperature for 30 minutes. The absorbance was measured by spectrophotometry at 517 nm, with butylated hydroxytoluene (BHT) as a reference. The IC_{50} value was determined by plotting the concentration of EEMO (*x*-axis) and DPPH inhibition absorbance (*y*-axis) into a linear regression. The resulting straight-line equation was used to calculate the IC_{50} value at $y = 50\%$.

Cytotoxicity test

The cytotoxicity potential of EEMO was studied in HepG2 and Madin–Darby canine kidney (MDCK) cells using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide) test by adopting the procedure from previous study (Abd-Rabou *et al*., 2017; Barhoi *et al*., 2021a). HepG2 and MDCK cells were cultured in a complete medium containing Roswell Park Memorial Institute (RPMI), 10% Fetal Bovine Serum, 1% penicillin–streptomycin, and 1% amphotericin B in a 5% CO_2 incubator. Briefly, 10.000 cells of HepG2 or MDCK were grown in 96-well plates and treated with various concentrations of MO extracts (ranging from 3.0 to 500 µg/mL) for 24 hours. Afterward, the cells were incubated with MTT reagents. The absorbance of the viable cells was then measured using an ELISA reader at a wavelength of 590 nm. The absorbance values were used to create a relationship curve between concentration and percentage of inhibition. From this curve, the concentration of MO extracts that inhibit 50% of cell viability (CC_{50}) was determined.

In vivo acute toxicity analysis in animals

An in-vivo acute toxicity analysis was conducted using experimental animals. Acute toxicity testing was conducted in accordance with the guidelines set forth by the Food and Drug Administration (Badan Pengawas Obat dan Makanan) Number 10 of 2022 regarding preclinical in vivo toxicity testing (BPOM, 2022).

Healthy Sprague–Dawley rats aged 8–12 weeks, consisting of 30 males and 30 females (150–200 grams), were utilized, ensuring a weight variation of no more than 20% within each group. Female rats selected for the study had never given birth and were not pregnant. The rats were obtained from the Animal Breeding Facility of the National Food and Drug Agency (BPOM). Prior to the experiment, the rats were acclimatized for one week in a controlled environment with a temperature of 22°±3° C, relative humidity of 30%–70%, and a 12-hours light/dark cycle. They were provided with standard laboratory feed and allowed access to water ad libitum. The test animals were fasted for 14–18 hours before treatment.

The rats were divided into five groups of six, each receiving different doses of EEMO: 5, 50, 300, 2,000, and 5,000 mg/kg BW, administered orally as a single dose. The test animals were observed individually for at least 30 minutes post-administration, with periodic checks every 4 hours during the first 24 hours, followed by daily observations for 14 days. Monitoring parameters included body weight (BW), skin condition, fur quality, eye appearance, mucous membranes,

respiration, autonomic nervous system, and central nervous system. On the final day of observation, all rats underwent necropsy under anesthesia using ketamine (35 mg/kg BW) and xylazine (5 mg/kg BW). Liver and kidney tissues were harvested and preserved in 10% formalin for histopathological analysis. The LD_{50} value was calculated according to BPOM Regulation No. 10 of 2022 (BPOM, 2022), based on the number of animal deaths and observed toxic symptoms.

Histological analysis

Tissue sectioning, paraffin block preparation, and tissue staining were performed at the Anatomic Pathology Laboratory, Faculty of Medicine, Universitas Indonesia. Liver and kidney tissue samples were collected and immediately preserved in 10% formalin for 24 hours. The dehydration process involved a series of graded alcohols, followed by clearing in xylene and paraffin block creation. The liver and kidney tissue sections were stained using hematoxylin and eosin (HE). All regions within the liver and kidney were observed at 10 High Power Fields (×40) under a binocular light microscope (LEICA® DM 1000 L30) and recorded using a SIGMA® HD microscope camera/HDMI/USB with the IndoMicroview 3.7 digital camera application.

Statistical analysis

IC_{50} and CC_{50} values were analyzed using Excel, employing linear regression of log concentration). The IC_{50} value was derived from the linear equation relating log concentration (*x*-axis) to the percentage of inhibition (*y*-axis) from three repeated measurements of the sample. Similarly, the CC_{50} value was obtained from the linear equation relating the log concentration of the test solution (*x*-axis) to the percentage of live cells (*y*-axis). Both IC_{50} and CC_{50} values were determined by substituting y = 50% into the linear regression equations. Differences in organ weights between the normal and treatment groups were analyzed using ANOVA, followed by Tukey's multiple comparison test for post-hoc analysis. All statistical analyses were performed using GraphPad Prism 10.2.2 for Windows.

Ethical approval

In this study, the ethical approval was received from the Ethics Committee, Faculty of Medicine, Universitas Indonesia (KET-640/UN2.F1/ETIK/PPM.00.02/2022, June 27, 2022).

Results

Optimal results of MO leaves extract in ethanol extraction using UAE method

The MO leaves used in this study were collected from the Kupang Regency in NTT. The duration of the extractions using the UAE method resulted in variations in protein content. Notably, the extraction of MO leaves from the Kupang Regency region using ethanol for 60 minutes yielded the highest protein content at 45.5%. Different durations of ethanol incubation were applied in this study, and the results showed that longer incubation times increased the protein yield. The net yield of MO extracts from dried leaves was 0.71%, with protein content ranging from 39.8% to 45.5% (Fig. 1). A proximate analysis was performed to compare the levels of protein, fat, carbohydrates, water, ash, and crude fiber in unextracted MO leaves powder and MO leaves extracts obtained from the optimal protein extraction using ethanol (EEMO) for 60 minutes with the UAE method. The results, shown in Table 1, indicate a difference in protein content between unextracted MO powder (28.76%) and EEMO (45.5%) extracted using the UAE method.

Examination of EEMO using standard test for herbal medicine

The extracted protein obtained from EEMO was examined using standard tests for herbal medicine. The results indicated that our extracts fully met the criteria for herbal medicine, including standards for aflatoxin levels, heavy metals, microbiological safety, moisture content (<10%), and ethanol content (<1%) (Table 2).

Determination of essential and non-essential amino acids from EEMO

The EEMO was analyzed to determine the contents of essential and non-essential amino acids (Table 3). Eight types of essential amino acids were found, with phenylalanine (22.25 mg/g), valine (13.09 mg/g), threonine (6.41 mg/g), and leucine (5.84 mg/g) presented in the highest concentrations. Additionally, eight types of non-essential acids were detected, with glutamic acid (60.03 mg/g) found in the highest concentration followed by aspartic (40.33 mg/g), arginine (14.69 mg/g) and alanine (13.50 mg/g).

Determination of minerals and phytic acid flavonoids content from EEMO

The EEMO was analyzed to determine its mineral content. Among the macro-elements, potassium was found to be the highest concentration (1,174.23 mg/100 g), followed by chloride and magnesium (Table 4). For the microelements, selenium was the most abundant element, with copper, zinc, and iron also presented in notable amounts (Table 4).

The results of the phytochemical analysis revealed that the major compounds were flavonoids, such as kaempferol-3,7-diglucoside, kaempferol-3-O-rutinoside, and kaempferol-7-O-α-L-rhamnoside, as well as phenyl propionic acid, as shown in the chromatogram (Fig. 2) and detailed in Table 5. To confirm the presence of phytic acid in EEMO, which does not inhibit nutrient absorption, we measured its level using spectrophotometry and a phytic acid standard series. The analysis indicated that the phytic acid content in EEMO was 13 mg/g.

Antioxidant potential with low cytotoxicity of EEMO

The antioxidant activity of EEMO was demonstrated with the IC_{50} 41.04 ppm, while ascorbic acid, used as the positive control of antioxidant agent, had an IC_{50} of 15.8 ppm (Fig. 3). The concentrations of EEMO needed to produce antioxidant activity were substantially lower than those required to induce cytotoxicity in HepG2

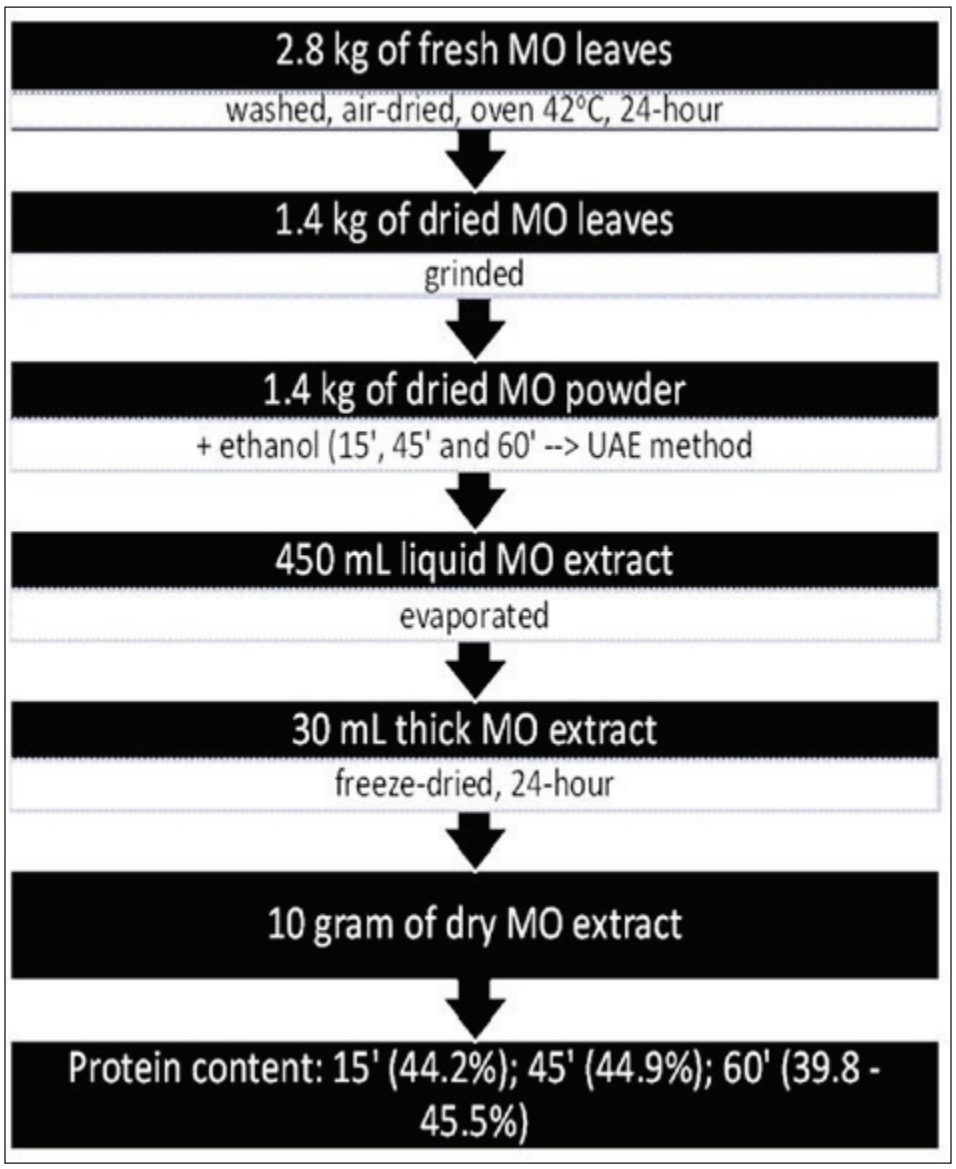


Fig. 1. The process of MO extraction from fresh leaves to freeze-dried extracts.

and MDCK cell lines. The cytotoxicity of EEMO was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cytotoxicity activity (CC_{50}) of HepG2 and MDCK were 182.66 and 121.04 ppm, respectively (Fig. 4a and b).

Safety of EEMO on test animals

The EEMO used in this study underwent acute oral toxicity testing at doses of 5, 50, 300, 2,000, and 5,000 mg/kg BW. The results indicated an LD_{50} value exceeding 5,000 mg/kg BW, with no signs of toxicity observed at the highest dose (Table 6). Throughout the 14-days observation period, no abnormalities were found in the skin, fur, eyes, mucous membranes, respiratory system, autonomic nervous system, and central nervous system**,** in any of the rats treated with

Table 1. Proximate analysis of MO leaves powder *versus* EEMO[a] from Kupang Regency.

Sample content	MO leaves powder	EEMO
Water, %	5.35	4.63
Ash, %	13.82	10.20
Carbohydrate, %	34.15	36.86
Protein, %	28.76	45.50
Crude Fat, %	7.49	1.10
Crude Fiber, %	10.94	1.81

[a]EEMO: Ethanol leaf extract of MO.

Table 2. Examination results of EEMO[a] following herbal medicine standard.

No.	Parameters	Unit	Content in EEMO	Quality standards[b]
1	Aflatoxin G2	µg/kg	ND[c]	≤ 20 µg/kg
2	Aflatoxin B2	µg/kg	ND	≤ 20 µg/kg
3	Aflatoxin G1	µg/kg	ND	≤ 20 µg/kg
4	Aflatoxin B1	µg/kg	ND	≤ 20 µg/kg
5	Total aflatoxin	µg/kg	ND	≤ 20 µg/kg
6	Water content	%	≤10%	≤10%
7	Ethanol content	µg/kg	<1%	<1%
8	Total plate content	colony/g	$\leq 10^5$colony/g	$\leq 10^5$colony/g
9	Yeast and mould	colony/g	$\leq 10^3$colony/g	$\leq 10^3$colony/g
10	*E. coli*	colony/g	≤10 colony/g	≤ 10 colony/g
11	*Enterobacteriaceae*	colony/g	$\leq 10^3$colony/g	$\leq 10^3$colony/g
12	*Salmonella* sp.	/g	negative/g	negative/g
13	*Shigella* sp.	/g	negative	negative/g
14	*Clostridium* sp.	/g	negative	negative/g
15	Arsenic (As)	mg/kg	ND	≤ 5 mg/kg
16	Mercury (Hg)	mg/kg	ND	≤ 0.5 mg/kg
17	Cadmium (Cd)	mg/kg	ND	≤ 0.3 mg/kg
18	Lead (Pb)	mg/kg	ND	≤ 10 mg/kg

[a]EEMO: ethanol leaf extract of MO, [b]: standard value was issued by the Indonesian National Agency for Food and Drug Control (PerKa BPOM no 32/2019). [c]: ND, not detected.

Table 3. Analysis results of amino acid composition in EEMO[a].

Type of amino acid	Concentrations (mg/g)
Essential amino acid	
Histidine	5.23
Isoleucine	4.99
Leucine	5.84
Lysine	3.00
Phenylalanine	22.25
Threonine	6.41
Tryptophan	2.04
Valine	13.09
Non-essential amino acid	
Glutamic acid	60.03
aspartic acid	40.33
Arginine	14.69
Alanine	13.50
Serine	8.12
Proline	7.07
Glycine	5.22
Tyrosine	3.07

[a]EEMO: Ethanol leaf extract of MO.

Table 4. Analysis result of mineral content in EEMO[a].

Mineral	Concentrations (mg/100 g)
Macro elements	
Chloride (Cl)	734.07
Potassium (K)	1174.23
Magnesium (Mg)	251.07
Calcium (Ca)	723.08
Microelements	
Zinc (Zn)	2.04
Iron (Fe)	1.90
Copper (Cu)	4.20
Selenium (Se)	149

[a]EEMO: Ethanol leaf extract of MO.

different doses of EEMO. No animals displayed severe pain or distress at the end of the study. The effect of EEMO on BW is shown in Figure 5. There were no significant differences in the average BW of male and female groups receiving different doses of EEMO compared to the control group (Fig. 5). Organs such as the brain, lungs, heart, liver, kidneys, spleen, and gut from all groups were harvested after euthanasia.

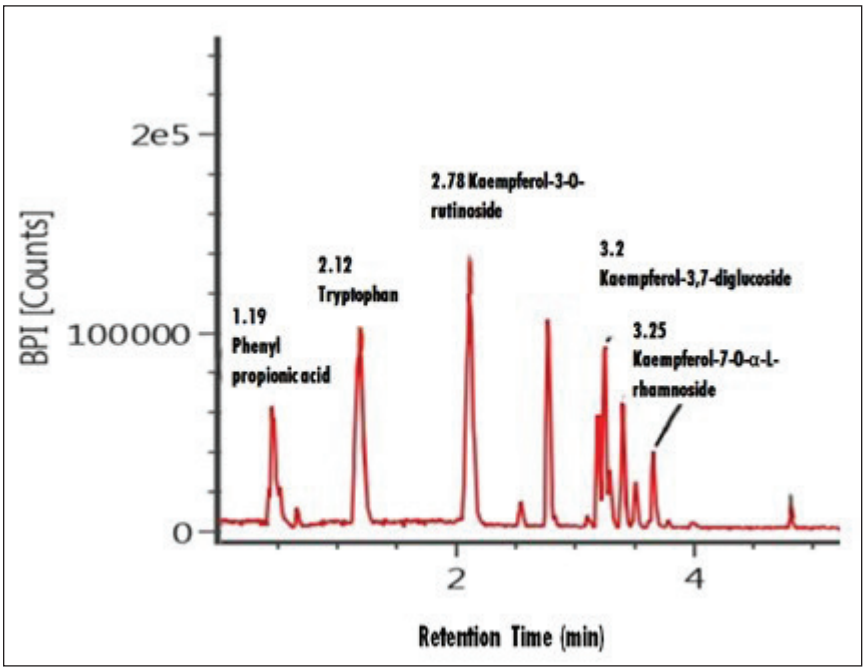


Fig. 2. LC-MS/MS chromatogram showing the presence of bioactive compounds in EEMO.

The organ weights of both male and female subjects in all EEMO treatment groups also did not significantly differ from those of the control group (Table 7).

HE staining of liver and kidney tissue

The liver and kidneys are crucial organs in drug metabolism; therefore, histopathological evaluations were focused on these tissues as shown in Figure 6. The liver histology results at all doses revealed no damage to the liver architecture (Fig. 6A). Similarly, the kidney histology results showed no damage to the architecture, though there was slight congestion of the hepatocytes in the treated groups (Fig. 6A and B).

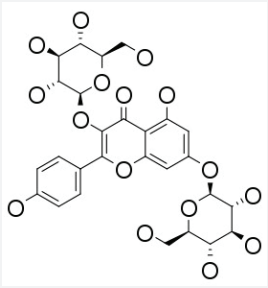
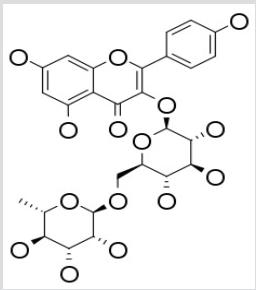
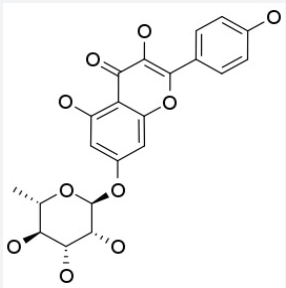
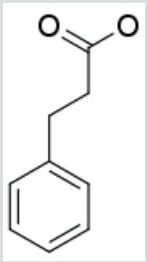
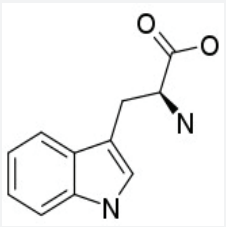
Discussion

Our study successfully extracted protein from MO leaves using the ultrasonic extraction (UAE) method with ethanol as the solvent, yielding up to 45.5% protein content. The ethanol-extracted MO (EEMO) exhibited high levels of flavonoids, complete amino acids, and complete minerals, and met the extract characterization standards set by the National Food and Drug Agency (BPOM). Our findings also showed that the toxicity dose of EEMO was six to seven times higher than the antioxidant dose on HepG2 cells and eight times higher on MDCK cells. The results of the acute oral toxicity test showed no death signs at the highest tested dose of 5.000 g/kg BW.

The UAE method has been developed as a cost-effective, low-temperature, solvent-efficient, environmentally friendly technique, with short extraction times and high yields for extracting bioactive compounds from different plant materials (Zheng *et al*., 2016). It is particularly effective in producing extracts rich in nutritional components and phytochemicals using various solvents (Garcia-Salas *et al*., 2010; Dadi *et al*., 2019b). The primary mechanism of UAE in releasing protein from cells is based on cavitation phenomena and mechanical mixing effects. Ultrasonic energy from the ultrasonic bath causes the cell walls of MO to break due to the explosion of cavitation bubbles on the surface of the MO powder. This facilitates mass transfer and allows for greater ethanol penetration into the cells of MO, increasing the contact surface area between the MO cells and ethanol, thus resulting in rapid solute diffusion from the solid phase to the solvent phase (Toma *et al*., 2001; Dranca and Oroian, 2016; Patil and Akamanchi, 2017).

Several studies have also reported the use of ethanol as a solvent for protein extraction, including ethanolic extract from *Anneslea fragrans* Wall (Wang *et al*., 2023), *Mentha piperita* leaf extract, and *Stevia rebaudiana* (Bert.) leaf extract (Zorzenon *et al*., 2023). The choice of solvent can significantly impact the quality of extraction and may even damage proteins. Both water and ethanol are recommended solvents for food processing by the National Food and Drug Agency (BPOM, 2023), food authorities in the USA, Australia, New Zealand, and the European Food Safety Authority (Carrillo-Álvarez, 2023). Ethanol has the advantage of a low boiling point, is easily separated from the product, and can dissolve proteins (Zhang *et al*., 2018; Yu *et al*., 2019; Lafarga *et al*., 2020). The use of ethanol in industry has also developed because it is inexpensive and relatively non-toxic compared to

Table 5. Phytochemical compounds found in EEMO[a].

No	Compounds	Chemical formula	Chemical structure	Observed m/z	Retention time (minutes)
1	Kaempferol-3,7-diglucoside	$C_{27}H_{30}O_{16}$		611.1633	3.20
2	Kaempferol-3-O-rutinoside	$C_{27}H_{30}O_{15}$		595.1684	2.78
3	Kaempferol-7-O-α-L-rhamnoside	$C_{21}H_{20}O_{10}$		433.1143	3.25
4	Phenyl propionic acid	$C_9H_{11}NO_2$		166.0862	1.19
5	Tryptophan	$C_{11}H_{12}N_2O_2$		205.0970	2.12

[a]EEMO: ethanol leaf extract of MO.

acetone and methanol. It can also be used for various extraction methods, and its extraction products are safe for medicines and food (Jiménez-Moreno *et al*., 2019; Chen *et al*., 2020).

MO leaves are known for their high protein. Several studies have reported protein content in MO leaves ranging from 23%–27% (D'Auria *et al*., 2023), depending on the part of the plant. Additionally,

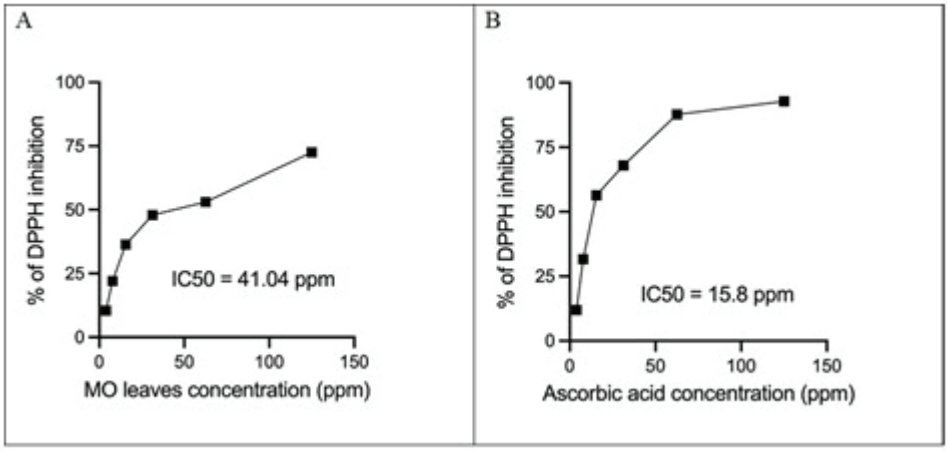


Fig. 3. Antioxidant activity of EEMO (A) compared to ascorbic acid (B) as a positive control. The antioxidant activity was measured using the DPPH radical scavenging assay, and the results are expressed as the percentage of DPPH inhibition.

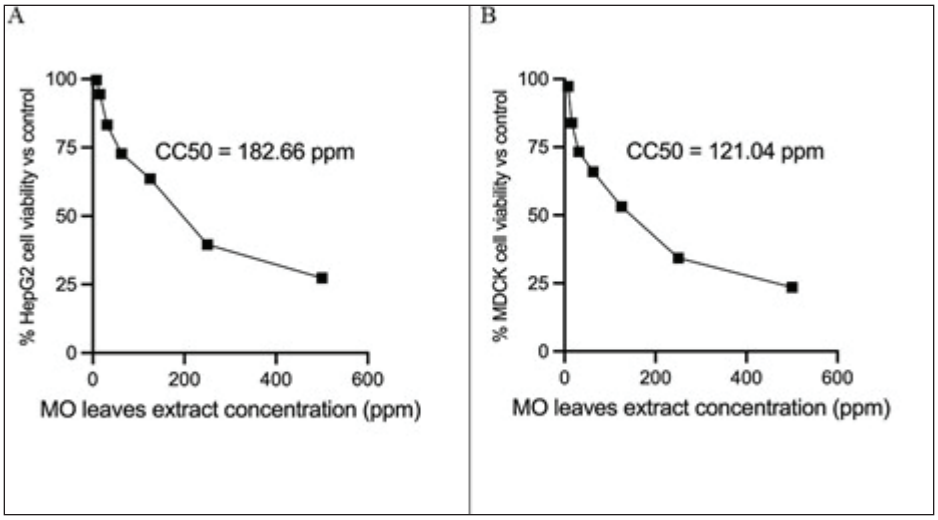


Fig. 4. Cytotoxicity concentrations of EEMO in (A) HepG2 and (B) MDCK cell lines.

Table 6. Acute toxicity (oral) in female and male mice 14 days after EEMO[a] consumption.

Group	Doses mg/kg BW	D/T[b]		Signs of toxicity observed
		Male	Female	
A	0.2 ml H_20	0/5	0/5	No toxic changes observed
B	5	0/5	0/5	No toxic changes observed
C	50	0/5	0/5	No toxic changes observed
D	300	0/5	0/5	No toxic changes observed
E	2,000	0/5	0/5	No toxic changes observed
F	5,000	0/5	0/5	No toxic changes observed

[a]EEMO: Ethanol leaf extract of MO; [b]D/T: Number of mice deaths/total number of mice (n = 5).

previous studies have reported the protein yield from MO leaves extracts from many areas ranging from 6.7%–33.82% (fresh leaves) (Sena *et al*., 1998; Reyes Sánchez *et al*., 2006), 24.2%–29.8% (dried leaves) (Hadju *et al*., 2021), 27.1% (leaves flour), and 2.5% (seeds) (Citra, 2019). Our extraction method, using the

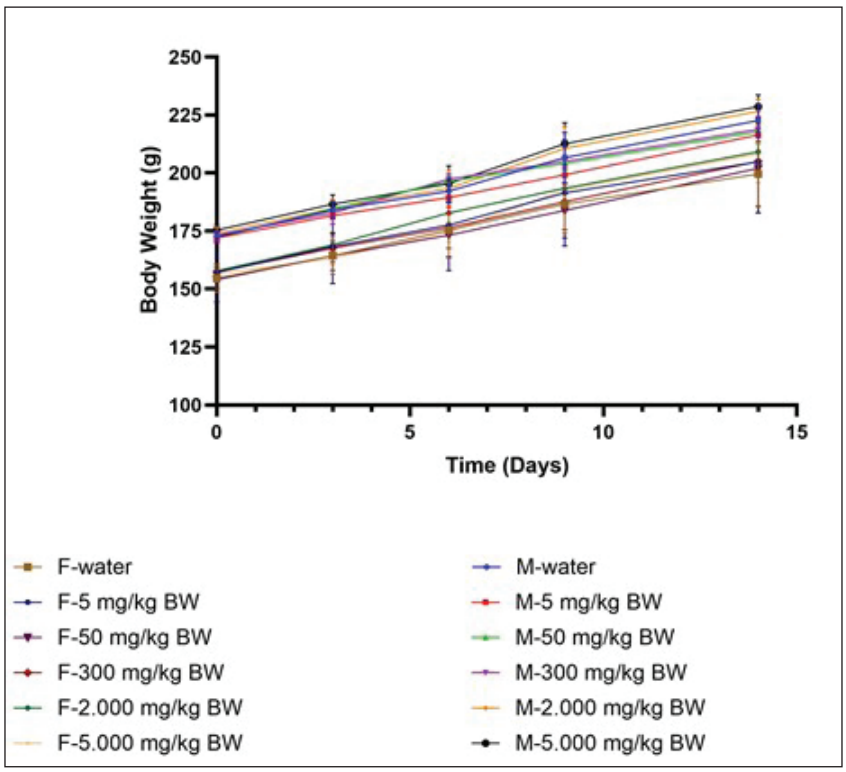


Fig. 5. The effect of EEMO on BW.

Table 7. Comparison of organ weights in male and female mice due to administration of various concentrations of EEMO[a].

Mice Sex	Organ	Dose of EEMO (mg/kg BW)					
		0	5	50	300	2,000	5,000
Male	Brain	1.79 ± 0.021	1.74 ± 0.065	1.71 ± 0.066	1.79 ± 0.054	1.76 ± 0.10	1.81 ± 0.07
	Lung	1.27 ± 0.171	1.17 ± 0.190	1.252 ± 0.078	1.49 ± 0.160	1.260 ± 0.129	1.34 ± 0.093
	Heart	0.79 ± 0.036	0.70 ± 0.055	0.73 ± 0.023	0.85 ± 0.094	0.76 ± 0.088	0.87 ± 0.052
	Liver	7.65 ± 0.590	6.70 ± 0.575	7.06 ± 0.580	7.87 ± 0.884	6.52 ± 0.530	8.73 ± 1.018
	Kidney	1.95 ± 0.100	1.56 ± 0.194	1.72 ± 0.113	2.07 ± 0.196	1.81 ± 0.108	2.05 ± 10.771
	Spleen	0.45 ± 0.111	0.45 ± 0.064	0.47 ± 0.046	0.50 ± 0.037	0.53 ± 0.066	0.56 ± 0.024
	Gut	20.17 ± 1.624	16.50 ± 0.616	17.9 ± 1.459	22.04 ± 3.165	21.64 ± 3.275	21.33 ± 2.431
Female	Organ	Dose of EEMO (mg/kg BW)					
		0	5	50	300	2,000	5,000
	Brain	1.61 ± 0.145	1.71 ± 0.074	1.67 ± 0.105	1.66 ± 0.107	1.76 ± 0.047	1.76 ± 0.051
	Lung	1.35 ± 0.167	1.29 ± 0.144	1.14 ± 0.245	1.20 ± 0.317	1.54 ± 0.131	1.57 ± 0.221
	Heart	0.74 ± 0.054	0.71 ± 0.103	0.68 ± 0.108	0.66 ± 0.031	0.75 ± 0.124	0.74 ± 0.090
	Liver	7.92 ± 0.365	7.60 ± 1.070	7.50 ± 1.141	6.68 ± 0.690	7.60 ± 0.614	7.40 ± 1.117
	Kidney	1.71 ± 0.152	1.56 ± 0.118	1.55 ± 0.176	1.56 ± 0.108	1.56 ± 0.174	1.54 ± 0.187
	Spleen	0.36 ± 0.112	0.55 ± 0.131	0.55 ± 0.190	0.44 ± 0.042	0.48 ± 0.071	0.46 ± 0.112
	Gut	22.75 ± 2.036	19.92 ± 4.076	21.66 ± 2.767	17.93 ± 3.037	21.81 ± 2.370	26.09 ± 3.657

[a]EEMO: Ethanol leaf extract of MO. Data are presented as the mean ± SD, median (range) (n = 5), Results were analyzed using O*ne-way* Anova, p value < 0.05, *post-hoc* test or tukeys *multiple comparisons test.*

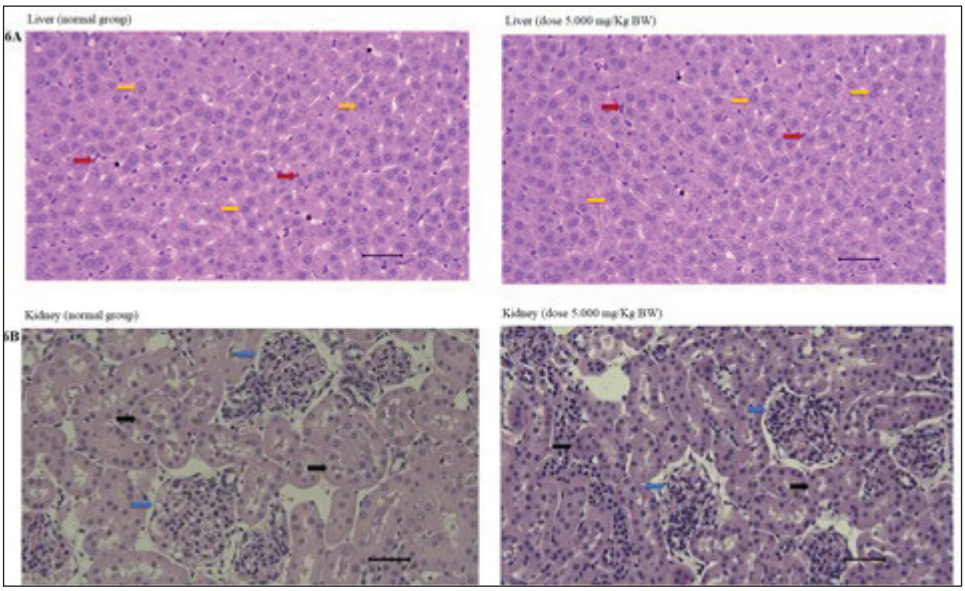


Fig. 6. Micrographs of the liver and kidney sections obtained from the normal group and the group treated with high doses of EEMO (5,000 mg/kg BB) are presented. Magnification: 400x. In (A), yellow arrows indicate the normal hepatocytes, red arrows indicate the Kupffer cells, and star shapes indicate the sinusoids. Both the normal group and the 5,000 mg/kg BB group show a normal structure of liver tissues. No degeneration or necrosis of the hepatocytes, cellular infiltration, or abnormalities were detected in these groups. Bar = 20 µm. In (B), blue arrows indicate the normal features of the renal glomerulus, and black arrows indicate the normal features of the renal tubules. Both the normal group and the 5,000 mg/kg BW group show a normal structure of kidney tissue. No degeneration or necrosis, cellular infiltration, or abnormalities were detected in these groups. Bar = 20 µm.

UAE extraction with ethanol solvent for 60 minutes, resulted in an optimal yield of protein obtained by ethanol extraction was 45.5% (Fig. 1).

According to National Food and Drug Agency standards (BPOM, 2022), several standard extraction methods are applicable for orally used products. Given the potential contamination of fresh MO leaves with heavy metals (Limmatvapirat *et al*., 2013; Grosshagauer *et al*., 2021) posing challenges in food processing, we conducted chemical and microbiological safety assessments on EEMO. Our findings indicated that EEMO produced using the UAE method met safety standards for physical, chemical, as well as metal and microbiological contamination (Table 2).

The high protein content in MO leaf extract had the potential to be a source of nutrition. Fresh MO leaves contain vitamins A, C, and B6, calcium, potassium, iron, and other nutritional content (Katmawanti *et al*., 2021). Several studies have reported that the nutritional content of Ca, Mg, Na, P, and K was 1.63%–2.2%, 0.36%–0.53%, 0.18%–0.43%, 012%–0.22%, and 0.67%–0.76%, respectively. Polyphenol and flavonoid levels varied between 0.24%–0.34% and 192–209 ppm (Wardana *et al*., 2022), respectively. The total amino acid content in MO leaves has been reported to range from 74.5 to 172.7 mg/g. The leaves contained the most amino acids, nearly double the amount found in the flowers. The amino acids in higher proportions were glutamic acid, arginine, and aspartic acid, while methionine and tryrosine were found in the lowest concentrations (Wu *et al*., 2013). Natsir *et al*. reported 15 amino acids, with the highest concentrations of essential and non-essential amino acids being leucine and glutamic acid, respectively (Natsir *et al*., 2019). Another study by Alhakmani *et al.* also reported 20 amino acids from MO leaves, with the highest proportions being aspartic acid, glutamic acid, leucine, arginine, and valine (Alhakmani *et al*., 2013).

Variations in amino acid composition can be influenced by protein quality and plant origin, whether cultivated or wild (Makkar and Becker, 1996; Sena *et al*., 1998). Our research identified complete amino acids in our extract, including essential amino acids such as phenylalanine, valine, threonine, and leucine, and non-essential amino acids such as glutamic acid, aspartic acid, arginine, and alanine (Table 3). Recent clinical nutrition studies emphasize not only total protein intake but also the balance and ratio of essential amino acids to total amino acids (Richard D. Semba *et al*., 2016a, b). The plant-derived amino acid content of EEMO is expected to complement the daily nutritional requirements of children and toddlers when combined with other animal-based foods.

Various studies reported the mineral content of MO leaves, with selenium and chromium being the highest minerals, while sodium and magnesium were found in lower levels (Moyo *et al*., 2011; Natsir *et al*., 2019). Other researchers also reported variations in mineral content, but almost all types of minerals were found in MO leaves (Mutar *et al*., 2021). Our investigation revealed that EEMO contained high levels of potassium, chloride, calcium, selenium, copper, and zinc (Table 4). Fortification of MO leaves in various food items, alongside the mineral content of EEMO in our study, is expected to complement the daily nutritional needs of children and toddlers (Govender and Siwela, 2020; Saeed *et al*., 2021; Alphonce *et al*., 2021).

MO leaves contain modest amounts of anti-nutrients such as saponins, phytates, and tannins, which may interfere with digestion and metabolic processes (Hadju *et al*., 2021). These compounds are produced by plants during regular metabolism and interact with the chemical composition or interfere with digestion and metabolic processes in the body through various pathways, leading to results that contradict healthy nutrition (Rahman *et al*., 2015). Saponins, tannins, flavonoids, alkaloids, protease inhibitors, oxalates, phytates, hemagglutinins (or lectins), cyanogenic glycosides, cardiac glycosides, coumarins, and gossypol are all well-known anti-nutritional compounds. The phytic acid concentration in MO leaves was observed to be lower than that of other household vegetables, with a reported content of 22.3 mg/g in dried leaves (Citra, 2019). Our investigation indicated that the amount of phytic acid in the ethanol extract was low (13 mg/g), which is unlikely to have significant anti-nutritional effects. In fact, the presence of phytic acid in low concentration may be advantageous, as it is known to display a broad range of pharmaceutical properties, including antioxidant, neuroprotective, anti-inflammatory, and lipid-lowering effects.

Aside from its nutritional value, our extract demonstrated antioxidant activity as measured by the DPPH test. DPPH test is a traditional and widely used in-vitro method for antioxidant activity assay due to its simplicity, speed, ease of use, sensitivity, and minimal sample requirements (Dadi *et al*., 2019a; Wang *et al*., 2023). This method also benefits from the use of stable DPPH compounds, as well as comparator compounds such as vitamins C, A, and E. The IC_{50} value in this approach measures the concentration of antioxidant compounds needed to inhibit 50% oxidation. The stronger the antioxidant activity, the lower the IC_{50} value.

Several studies reported the antioxidant activity of MO leaves, attributing it to active compounds such as polyphenols, flavonoids, phenolics, and carotenoids. Several extraction methods have been used to extract antioxidant compounds, including the UAE method (Desmarchelier *et al*., 1997; Hamed *et al*., 2019; Zorzenon *et al*., 2023), subcritical water extraction (SWE), SFE, microwave-assisted extraction (MAE). The antioxidant compounds were also found in EEMO extracted by the UAE method in this study. The phytochemical analysis of EEMO revealed the presence of, several compounds expected to contribute to antioxidant activity, including kaempferol-3,7-diglucoside, kaempferol-3-O-rutinoside, and kaempferol-7-O-α-L-rhamnoside. These three compounds are flavonoid derivatives known for their strong antioxidant properties (Table 5).

The antioxidant activity of EEMO, as measured by the IC_{50} value, was 41.04 mg/l, with vitamin C as the standard, which had IC_{50} of 1.58 mg/l (Fig. 3a and b). Previous studies have reported various IC_{50} values for antioxidant activity in MO leaf extracts. The IC_{50} value of dichloromethane extracts in MO leaves were 1.60 ± 0.03 mg/ml in the DPPH test and 1.02 ± 0.06 mg/ml in the ABTS test (Suphachai, 2014). Wang *et al* reported the IC_{50} value of the MO extract was 0.7440 mg/l, compared to OPC as a standard which had IC_{50} value of 0.0195 mg/l (Wang *et al*., 2017).

To investigate the potential toxicity of EEMO, we used the MTT test to assess its cytotoxicity. The MTT Assay is a technique for determining a compound's cytotoxicity against cells. The reduction of MTT to formazan crystals is the concept of the MTT test, which is based on the capacity of the cellular enzyme oxidoreductase to convert nicotinamide adenine dinucleotide (NADPH) into insoluble formazan with a purple color. Formazan will be created by live cells that have an active metabolism (Abd-Rabou *et al*., 2017; Barhoi *et al*., 2021a).

We have tested our sample on two cells. HepG2 and MDCK cells, determined the CC_{50} values to be 181.66 and 121.04 mg/l, respectively (Fig. 4a and b). HepG2 cells are a hepatocarcinoma cell line that has been successfully cultivated. These cells have a high degree of morphological and functional differentiation. They are more stable and uniform than primary liver cells, making them suitable as a model for studying human protein dynamics in vitro. Moreover, HepG2 cells can produce CYP3A4 enzymes which metabolize more than 50% of drugs (Iskandar mudasyah *et al*., 2012; Sae-be *et al*., 2023). MDCK cells are known to be widely used for drug elimination, permeability, and drug solubility tests, making them useful for assessing the potential toxicity of MO extracts (Varma *et al*., 2012; Hosey and Benet, 2015). Our study showed that the toxicity dose of EEMO was 6–7 times higher than the antioxidant dose in HepG2 cells and 8 times in MDCK cells (Figs. 3 and 4).

The acute oral toxicity test of EEMO indicated that the highest dose of 5,000 mg/kg BW did not show any signs of toxicity or mortality in the test animals (Table 6). This result suggests that EEMO is safe for oral consumption (non-lethal) in acute exposure. Previous

studies have reported toxicity tests on MO in various extract forms, including methanol MO extract, which was found to be toxic at doses of 1,600 and 3,200 mg/kg BW (Adedapo *et al*., 2015). However, the methanol leaf extract of MO did not show toxic signs of death at doses >4,500 mg/kg BW, and the protein isolated from MO (MO-LPI) was reported to be non-toxic at a dose of 2,500 mg/kg BW (Omodanisi *et al*., 2017).

The histopathological examination of liver and kidney tissues (Fig. 6) did not reveal any differences between the normal group and the group administered the highest dose (5,000 mg/kg BW) of EEMO in both, male and female rats. Furthermore, no remarkable lesions were found in the examined organs. These findings indicate that EEMO does not induce histological lesions in the liver and kidney.

Conclusion

The ethanol leaf extract of MO (EEMO) obtained through the UAE method at 40° for 60 minutes meets safety standards and exhibits high nutritional value, including a rich protein and amino acid composition, balanced minerals, and low phytic acid content. It is also a potent natural source of antioxidants with non-cytotoxic properties. No acute toxic effects were found in the animals, and histological examinations revealed no lesions. Additionally, there was no effect of EEMO on BW and organ weight, indicating the safety of the extract as a source of nutrition and medicinal ingredients.

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Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

NTK conceptualized the study, and performed sample collection, laboratory work, data management, analysis, and manuscript writing. ML participated in conceptualized the study, manuscript writing, and data analysis. PEK participated in the result interpretation. OSMS participated in manuscript writing and editing. TS participated in study supervision, and manuscript editing. All authors read and approved the final manuscript.

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Data availability

All data supporting the findings of this study are available within the manuscript.

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