Journal of Tropical Forest Science 37(Special Issue): 20–40 (2025)
https://doi.org/10.26525/jtfs2025.37S.SI.20
ISSN: 0128-1283, eISSN: 2521-984

IN VITRO GASTROINTESTINAL DIGESTION ON THE ANTIOXIDANT ACTIVITY OF BIOACTIVE COMPOUND IN HYDROETHANOLIC LEAF EXTRACT OF *MORINGA OLEIFERA*

Khadijah Nabilah MZ, Mazura MP*, Shalini M, Khoo MGH & Juliza M

Natural Products Division, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor, Malaysia

**mazura@frim.gov.my*

Submitted May 2024; accepted December 2024

Moringa oleifera (MO) is a medicinal and nutritional plant rich in bioactive compounds, macro- and micronutrients that are important for prevention or treatment of diseases and sustaining healthy body function. Almost all parts of this plant are edible and possess therapeutic properties, such as antioxidant, antidiabetic, anticancer, and antimicrobial effects. This study aimed to investigate the antioxidant activity and polyphenol content of *M. oleifera,* and to evaluate its bioaccessibility and residual antioxidant activity during *in vitro* digestion. Changes in antioxidant activity were evaluated through DPPH, ABTS, TPC, TFC, FRAP, ORAC, SOD and CUPRAC assays in the oral, gastric, and intestinal phases. The cytotoxic effect of MO was evaluated using MTT assay against Vero and WRL-68 cell lines. The antioxidant activity of the MO leaf extract during simulated in vitro digestion was the highest at the stage of oral digestion, with the ABTS test showing the highest residual antioxidant activity (84.52 ± 3.98%). Overall, the digestive process affected the bioaccessibility and residual antioxidant activity of the MO leaf extract, which decreased in every phase on most of the assays tested. In the cytotoxicity test, the MO leaf extract had high IC_{50} values (> 1000 µg/mL), indicating low toxicity against all tested cell lines. These findings suggest that *M. oleifera* possesses high antioxidant properties and remains bioaccessible across all phases of digestion, which may have potentially beneficial effects on health.

Keywords: *Moringa oleifera, in vitro* digestion, antioxidant, bioaccessibility, residual antioxidant activity, cytotoxicity

INTRODUCTION

Moringa oleifera (MO) the "drumstick tree" that is also known as the "Tree of Life", or "Miracle Tree" is a valued plant that is widely distributed geographically (in the tropics and subtropics). MO is the most extensively cultivated species of the monogeneric family of shrubs and trees of Moringaceae, especially in sub-Himalayan countries such as India, Africa, Sri Lanka and Madagascar (Saini et al. 2016). The plant is easy to propagate and the ability of the plant to adapt to different soils and climate change is remarkable. Hence, it can be a good alternative for reducing hunger and malnutrition issues since it has gained popularity in various sectors especially in the agricultural sector (Trigo et al. 2021). MO has a high nutritional content, particularly its leaves. In general, it comprises over 90 nutritional bioactive compounds, including all macronutrients and phenolics (Gopalakrishnan et al. 2016). Major compounds in MO leaves are carotenoids, tocopherols (α, β and γ), flavonoids, phenolic acid, folate, various minerals and fatty acids (polyunsaturated), with small amount of saturated fatty acids (Saini et al. 2016, Kashyap et al. 2022, Padma et al. 2022).

Moringa oleifera has an impressive range of medicinal uses, and every part of this plant is considered an important element. In addition, researchers reported that this plant has many therapeutic applications in the treatment of diseases, especially in the traditional medicinal practices. Generally, MO leaves can be used for the treatment of skin diseases (acne, itchy skin, eczema), flu, asthma, diarrhoea, malaria, bronchitis, scurvy, headaches, heart burn, pneumonia, syphilis, dyslipidaemia and hyperglycaemia. Also, it has anticancer, antimicrobial, antioxidant, antidiabetic, antiulcer, antipyretic, anticonvulsion and antiatherosclerotic properties (Goyal et al. 2009, Jung et al. 2015, Paula et al. 2017, Othman et al. 2019, Abdallah et al. 2023). This MO

leaf is traditionally used and consumed fresh, or it can also be cooked and stored at room temperature as dried powder without affecting the nutritional values for a few months (Migala 2022). Nowadays, MO has been incorporated into different foods, such as ingredient of soup, paneer, chocolate and pastries (Farzana et al. 2016, Hedhili et al. 2021, Aly et al. 2022).

Antioxidant activity of MO is primarily attributed to the availability of quercetin, ascorbic acid, rutin, kaempferol, polyphenols and B carotene (Bajpai et al. 2005, Amaglo et al. 2010, Vats & Gupta et al. 2017, Barzan et al. 2022). The optimal gradient solvent for MO leaves was found to have the highest antioxidant activity in the water:ethanol extract using a ratio of 10:90, and this product was in the form of volatile oil obtained from distillation process of MO leaves (Karthivashan et al. 2013). The major bioactive constituents present in this hydro-ethanolic extract were quercetin, apigenin and kaempferol. Solvent extraction using methanol and acetone has also been found to exhibit antioxidant activities through the DPPH and ABTS tests (Moyo et al. 2012, Charoensin 2014,). Other phenolic compounds identified in the aqueous extract were epicatechin, ferulic acid and myricetin (Tai et al. 2018).

Despite the importance of bioactive compounds for antioxidant activity from MO leaves, studies on the impact of *in vitro* gastrointestinal digestion on these compounds are still sparse. The stability of antioxidant compounds during simulated *in vitro* digestion represents an important area of investigation that is crucial to assessing their potential bioactivity. Therefore, it is important to study the accessibility of these compounds for absorption and their ability to influence biological responses during digestion. Apart from that, *in vitro* bioaccessibility towards bioactive compounds release in the gastrointestinal tract from solid or food matrices and their stability are also worthwhile to be explored (Tagliazucchi et al. 2010).

The effectiveness of bioactive compound absorption through human digestive system is influenced by the action of digestive enzymes; which include amylase, pepsin, pancreatin and trypsin (Hur et al. 2011). Over the years, *in vitro* digestion models have been performed as the most common technique for the primary screening of the behaviour of the chemical composition and allow determination of bioaccessibility of bioactive compound in food uptake (Versantvoort et al. 2005, Akter et al. 2022). *In vitro* gastrointestinal digestion models simulating the physiochemical processes are comparatively simple (conditions can be effectively controlled), reasonable, rapid and less ethical constraints (Mackie et al. 2020, Ji et al. 2021). Since many previous studies have identified antioxidant compounds of activities in plant, but these studies have disregarded the influence of digestion. Thus, these considerations prompted us to perform the *in vitro* human digestion model study at different stages and allow the determination of the amount of bioaccessible bioactive compound and their antioxidant activity through digestion processes. The possibilities of both antioxidant activity and bioaccessibility index decline after the process of simulated digestion, which is a critical aspect for evaluating the actual potential use of MO leaves.

MATERIALS AND METHODS

Plant material and sample preparation

Moringa oleifera leaves (50 g) were collected from Semenyih in December 2021. The plant sample was verified by a botanist of Forest Research Institute Malaysia. Voucher specimen (SBID: 044/21) was prepared and deposited in Natural Products Division, Forest Research Institute Malaysia for future reference. The plant material was cleaned and dried in the oven at 40°C. Dried MO leaves were mixed with a 1:1 ratio of ethanol and distilled water for one day on rotary shaker at room temperature. The water-solvent was changed daily for three times and filtered using a Whatman filter paper. Then the hydroethanolic extract was dried and concentrated on a rotary evaporator to remove the solvent from the extract.

Sample analysis

The phytochemical, microbial and microscopy analyses of plant extracts were carried out according to in-house standard procedures. This includes: 1) determination of phytochemical constituents' (alkaloids, flavonoids, saponins,

steroids, tannins and triterpenes); 2) microbial contamination test on MO leaf extract using two different testing methods (microbial enumeration tests and specified microorganisms tests); 3) heavy metal analysis and 4) plant microscopic identification.

In vitro gastrointestinal digestion

The *in vitro* gastrointestinal digestion was performed based on the INFOGEST 2.0 procedure. The simulated salivary fluid (SSF) simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the procedure (Brodkorb et al. 2019) as shown in Table 1. The *in vitro* digestion tested in this study was performed using a static model through single and multiple phases.

Single-phase static model is generally a single-step digestion process, where digestion process was separately tested for each stage of digestive system. Briefly in oral stage, 1 mg of sample extract was suspended in 1 mL of SSF and mixed with a mixture of 1% of potato starch solution and 80 U/mL of α-human amylase in a 1:1 volume ratio, then it was incubated at 37 °C for 3 min. The oral enzymatic reaction was tested through DNS test, where the reaction was terminated by cooling the sample in a cooling bath for 15 minutes after being boiled at 100°C (Menard et al. 2023). Next, in the gastric stage, 100 µL of both 2000 U/mL of porcine pepsin and 200 U/mL of rabbit gastric extract solution were added to the mixture of sample extract and SGF solution. The sample mixture was incubated for 2 hours and 30 minutes at 37°C in a rotary incubator by constantly maintaining a pH of 3.0. Then, in order to stimulate the intestinal condition, the same concentration of plant extract was introduced to SIF and 8 mM of bile salt and pancreatin with trypsin activity of 100 U/mL. The pH was verified and adjusted to 7, and the sample was incubated for 3 hours at 37°C. All sample digesta in all stages of the digestive system were terminated by cooling the sample in the ice bath for 15 minutes, and centrifuged for 10 minutes at 10,000 g. The supernatant was separated immediately to ensure the stability of the antioxidant compounds in the mixture and stored at -20°C for further analysis.

In multiple phase, about 700 µL of sample digesta in every stage (oral, gastric, intestine) was further digested successively in the next stages. A similar procedure was applied in the multiple phases of gastrointestinal digestion, using the same volumes, concentrations and conditions of simulated solutions, enzymes and proteins as in the single-phase digestion.

Quantification of antioxidant activity

The antioxidant activity of soluble fractions obtained during the gastrointestinal digestion as post-digestion samples (oral-single and multiple phase (OSM), gastric-single (OS), intestinal-single (IS), gastric-multiple (GM), intestinal-multiple (IM)) and plant extract as undigested sample was evaluated by different type of assays; DPPH, ABTS, TPC, TFC, FRAP, ORAC, SOD and CUPRAC.

2,2-diphenyl-1-picrylhydrazyl (DPPH)

DPPH free radical scavenging assay was carried out with some modification of method from Blois (1958). The sample extract or standard (1 mg/mL), DPPH (in 1 mM solution of ethanol) and ethanol (absolute, AR Grade) was mixed in microplate with ratio of 1:1:3. The mixture was incubated for 30 min at room temperature before reading at 520 nm using spectrophotometer. The radical scavenging activity was determined using the formula below.

$$\text{DPPH}\% = \frac{\text{Negative control} - \text{Sample}}{\text{Negative control} - \text{Positive control}} \times 100$$

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonid acid) (ABTS)

The ABTS radical quenching assay was performed following Ozgen et al. (2006) with minor modifications. About 900 µL of ABTS solution was added to 100 µL of Moringa leaf extracts in 96-microtiter plate, and the absorbance was read at 734 nm using the FLUOstar Omega spectrophotometer. The ABTS radical scavenging activity is measured as follows:

$$\text{ABTS}\% = \frac{\text{Negative control} - \text{Sample}}{\text{Negative control}} \times 100$$

Table 1 Preparation of stock solutions of simulated digestion fluids. The final volume for each digestive fluid is 500 mL at a concentration of 1.25 ×

Constituent	Stock conc.		SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
	g/L	Conc. (M)	Volume of stock (mL)	Final concentration (mM)	Volume of stock (mL)	Final concentration (mM)	Volume of stock (mL)	Final concentration (mM)
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	11.8	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.4	1.1	0.33
$(NH_4)CO_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
HCl	-	6	0.09	1.1	1.3	15.6	8.4	8.4
$CaCl_2(H2O)_2$	4.41	0.3	0.025	1.5	0.005	0.15	0.6	0.6

Abbreviation: Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

Total phenolic content (TPC)

This assay was performed according to the Singleton and Rossi (1965) using Folin-Ciocalteu's reagent with modifications into high-throughput microplate system. About 50 µL of extract (1 mg/mL) was mixed with 100 µL of Folin-Ciocalteu's reagent (0.1/0.9 mL) in a 96 well microplate and incubated for 5 min at room temperature. Then, 100 µL of sodium bicarbonate (60 mg/mL) solution was added in the well and continued incubate for 90 min at room temperature. Absorbance was measured at 725 nm, and gallic acid was used as standard phenol.

Total flavonoid content (TFC)

TFC was evaluated using colorimetric method (Woisky & Salatino 1998), where 1.5 mL of 2% aluminium trichloride (AlCl3) was mixed with 1.5 mL of diluted extracts (500 ppm in 80% absolute ethanol). After 1 h incubation at room temperature, the absorbance was read at 420 nm against a blank. Quercetin compound was used to make the calibration curve.

Ferric reducing antioxidant (FRAP)

The FRAP assay was carried out based on the method of Musa et al. (2011). FRAP reagent consists of; 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tri(2-pyridyl)-striazine (TPTZ) and 20 mM $FeCl_3$·6H2O in the ratio of 10:1:1. About 100 µL sample or standard was mixed with 3.9 mL of FRAP reagent (freshly prepared and warmed at 37°C). Finally, absorbance was measured at 595 nm after 30 min of incubation.

Oxygen radical absorbance capacity (ORAC)

The ORAC assay was executed as reported by Huang et al. (2002) with some adjustments. All chemical stock solutions in this assay were freshly prepared before use. About 0.65 g of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) solution was dissolved in 10 mL of 75 mM phosphate buffer (pH 7.5). A fluorescein stock solution (1 mM) was made in phosphate buffer pH 7.4 (75 mM) and wrapped in foil at 5°C before stored. Prior to use, the stock solution of fluorescein was diluted 1:100 000 with phosphate buffer pH 7.4. For each microplate well, 150 µL of fluorescein working solution was added, while the sample wells received 25 µL of samples. The blank wells received 25 µL of Trolox dilution. The plate was then incubated for 10 minutes at 37 °C for equilibration purposes. A BMG Omega FLUOstar fluorescent spectrophotometer with injector was used with an excitation filter of 485 nm bandpass and an emission filter of 528 nm bandpass. To initiate the chemical reaction, the addition of 25 µL of APPH solution (240 mM) using the microplate reader's injector (final volume 200 µL) was done, followed by shaking at maximum intensity for 50 sec. The fluorescence was then observed kinetically with data taken in each well was measured by top reading every 60 sec. ORAC values were calculated using MARS Data Analysis Reduction Software.

Superoxide dismutase (SOD)

The assay method by Chang et al. (1996) was lightly modified to evaluate extract scavenging activity against superoxide-free-radical anions. NBT solution (4.1 mM, 100 mL) was prepared by dissolved Tris-HCL (3.15 g), MgCl2 (0.1 g), 5-bromo-4-chloro-3-indolyl phosphate (15 mg) and 4-nitro blur tetrazolium chloride (34 mg). Then, 4.0 mg EDTA, 50.0 mg xanthine and 0.53 g Na_2CO_3 (pH 10.2) were dissolved in a 0.025 mM NBT solution to make the reaction mixture (100 mL). The mixture was refrigerated at 4°C, prior to use. Meanwhile, the extracted sample was dissolved in the reaction mixture, resulting in concentrations ranging from 1.56 to 200 g/mL. After that, 50 mL of stock solution was added to 180 mL of the reaction mixture, followed by 20 mL of xanthine oxidase (20 U/mL). FLUOstar OMEGA spectrophotometer measures absorbance at 560 nm. The formula of superoxide scavenging activity as shown below.

$$\text{SOD}\% = \frac{\text{Negative control slope} - \text{Sample slope}}{\text{Negative control slope}} \times 100$$

Cupric ion reducing antioxidant capacity (CUPRAC)

The reducing antioxidant capacities of the extracts were investigated using CUPRAC assays. The CUPRAC assay was performed according

to the adapted method described previously (Özyürek et al. 2012). The CUPRAC reagent was prepared by mixing 10 mM of copper (II) chloride, 7.5 mM of neocuproine, and 1 M of ammonium acetate buffer with a pH of 7.0. Extract, water, or Trolox (50 µL) was mixed with 1.0 mL of CUPRAC reagent and allowed to stand in the dark for 30 minutes before the absorbance was measured at a wavelength of 450 nm. The samples were prepared at a concentration of 1000 ppm, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as the standard. The absorbance of the sample mixtures was read via spectrophotometer (BMG Labtech, Germany). The result was recorded as mg of Trolox equivalents (TEs) per g of extract (mg TE/g).

Antioxidant bioaccessibility index and residual antioxidant activity

The following indices were developed to help us better understand how antioxidants and their bioaccessibility in organic systems relate to one another. The antioxidant bioaccessibility index and residual antioxidant activity was calculated as follows:

$$\text{Antioxidant bioaccessibility index}\,(\%) = \frac{\text{Content in BF}}{\text{BC}} \times 100$$

$$\text{Residual antioxidant activity}\,(\%) = \frac{\text{AA}}{\text{BC}} \times 100$$

where BF and AA refers to bioaccessible fraction and antioxidant activity that was quantified in each sample at each simulated digestion stage, respectively. BC is the bioactive compound content or antioxidant activity quantified in MO leaf extract at the start before digestion (de la Fuente et al. 2019, Qin et al. 2022). Antioxidant bioaccessibility index and residual antioxidant activity were calculated as percentage in the digestion process at each stage compared to the activity of the MO before digestion, respectively.

In vitro toxicity (cytotoxicity)

Vero (African Green Monkey kidney) and WRL-68 (human liver) cell lines were obtained from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. The cells were maintained at 37 °C and 5% CO_2/95% air. For experimentation, cells growing at exponential were seeded in a 96-well plate at the density of 1×10^4 cells/well in complete culture medium (100 µL/well). After an overnight incubation, the culture medium was removed and cells were washed with phosphate buffered saline (PBS) (150 µL/well). Then, the cells were treated with various concentrations of the test samples (0–1000 µg/mL) in serum-free medium for 24 hours. The test samples were dissolved in dimethyl sulphoxide (DMSO) before further diluted in serum-free medium. The final concentration of DMSO in the test system was maintained at 0.5%. Sodium lauryl sulphate (0–60 µg/ml) was used as the positive control. After the exposure incubation period, cell viability was assessed by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay based on Mosmann (1983) with slight modifications. Percentage of viability was calculated by dividing the blank-corrected absorbance of sample with blank-corrected absorbance of the negative control. Dose-response curve was then plotted and the 50% inhibitory concentration (IC_{50}) was determined from the dose-response curve by non-linear regression using GraphPad Prism 8 software.

Statistical analysis method

All tests in this study were conducted in triplicate and the data are expressed as the mean ± standard error mean. The data was evaluated using GraphPad Prism 8 through Tukey's tests. One-way analysis of variance (ANOVA) was followed, where a p value less than 0.05 is considered statistically significant.

RESULTS

Sample analysis

Results on phytochemical, microbial and microscopy analysis of MO leaf extract are summarised in Table 2. Qualitative phytochemical analysis revealed the presence of tannins, flavonoids and steroids in plant extract. The formation of brownish green signifies the detection of condensed tannins in the MO leaf extract. Meanwhile, weak

colour appeared in the flavonoid test (yellow colour in ammonia layer) and steroids test (greenish colour using Liebermann-Buchard reagent). Other phytochemical constituents like alkaloids, saponins and terpenes have also been tested in this study, however these compounds were absent in the extracted leaves of MO. Meanwhile, in the microbial test, both total aerobic microbial count (TAMC) and total combined yeasts count (TYMC) methods used in this study passed the maximum acceptable count.

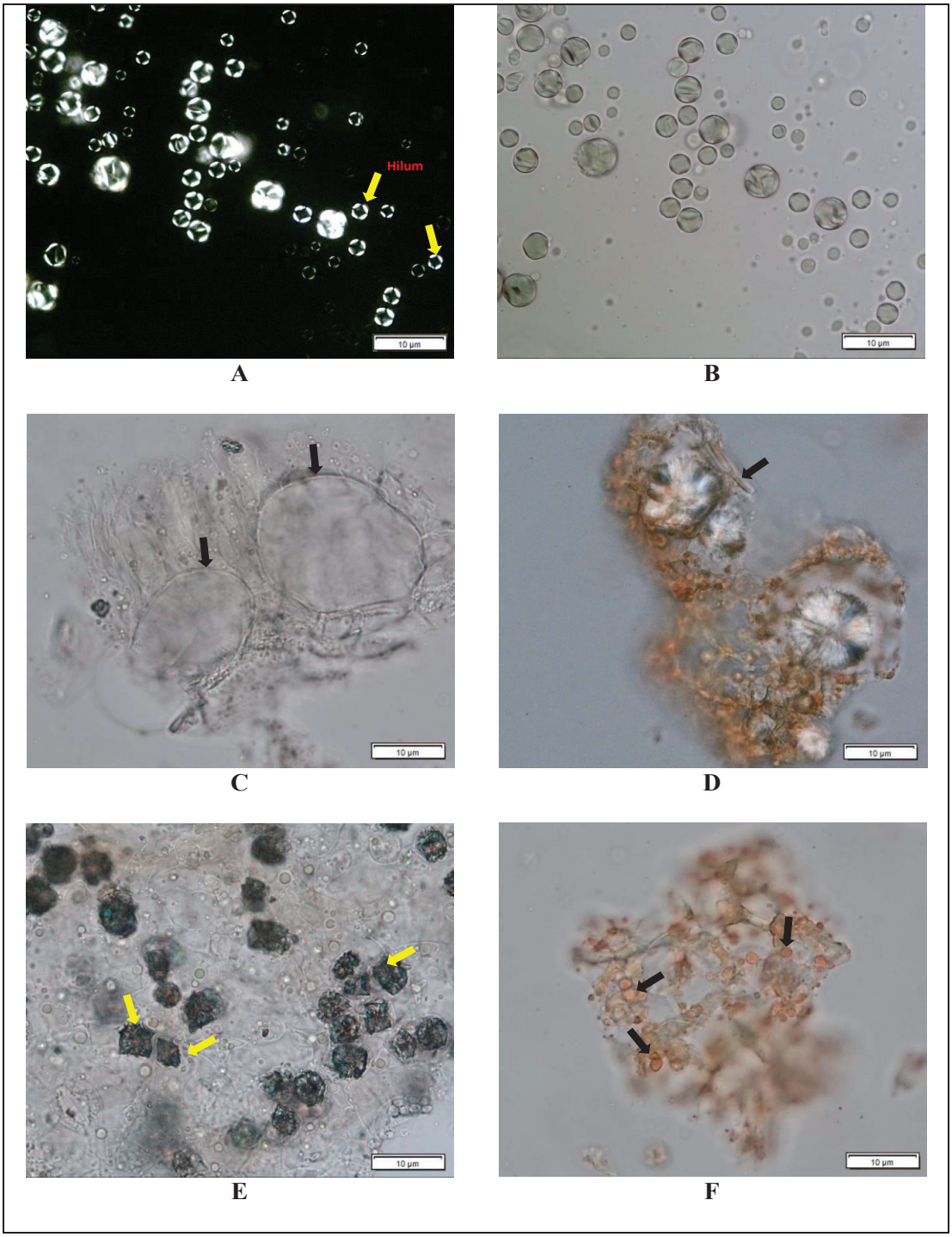
Four heavy metals tested in this study (Pb, Cd, Hg and As) exhibited low concentrations in MO leaf extract as shown in Table 2. The ranking of toxic heavy metals in the plant extract was Pb> As > Cd > Hg. All metals tested exhibited less than the maximum limit of heavy metals as suggested by the Drug Registration Guidance Document (DRGD) National (NPRA 2022), where it must not exceed 10.0, 0.3, 0.5 and 5.0 ppm for Pb, Cd, Hg and As, respectively.

Table 2 Chemical and microbial analysis of *Moringa oleifera* leaf extract

Parameters	Properties
Phytochemical compound	Flavanoids (1+), tannins (2+), steroids (1+)
Microbial enumeration tests:	
Total aerobic microbial count (TAMC)	1.40 x 10^5 CFU/g
Total combined yeasts/mould count (TYMC)	2.00 x 10^5 CFU/g
Test for specified microorganisms:	
Escherichia coli	<10 PN/g
Salmonella spp.	Absent
Heavy metal analysis:	
Lead (Pb)	0.50 mg/kg
Cadmium (Cd)	0.13 mg/kg
Mercury (Hg)	0.01 mg/kg
Arsenic (As)	0.50 mg/kg

1+: weak, 2+: mild

Determination of the identity and morphological of the plant material was done through microscopic evaluation. A microscopic profile of the transection of MO leaf extract was shown in Figure 1, where there was the presence of primary metabolites which are carbohydrate (starch grains with a rounded shape and hilum at the centre), idioblast cells containing oils and calcium oxalate crystals, and basic plant tissues (such as parenchyma cells). Other characteristics observed included fragments of adaxial and abaxial epidermal cells with sinous anticlinal walls and anomocytic stomata cells fragments of xylem elements (such as scalariform and spiral vessels) and simple and unicellular trichomes.



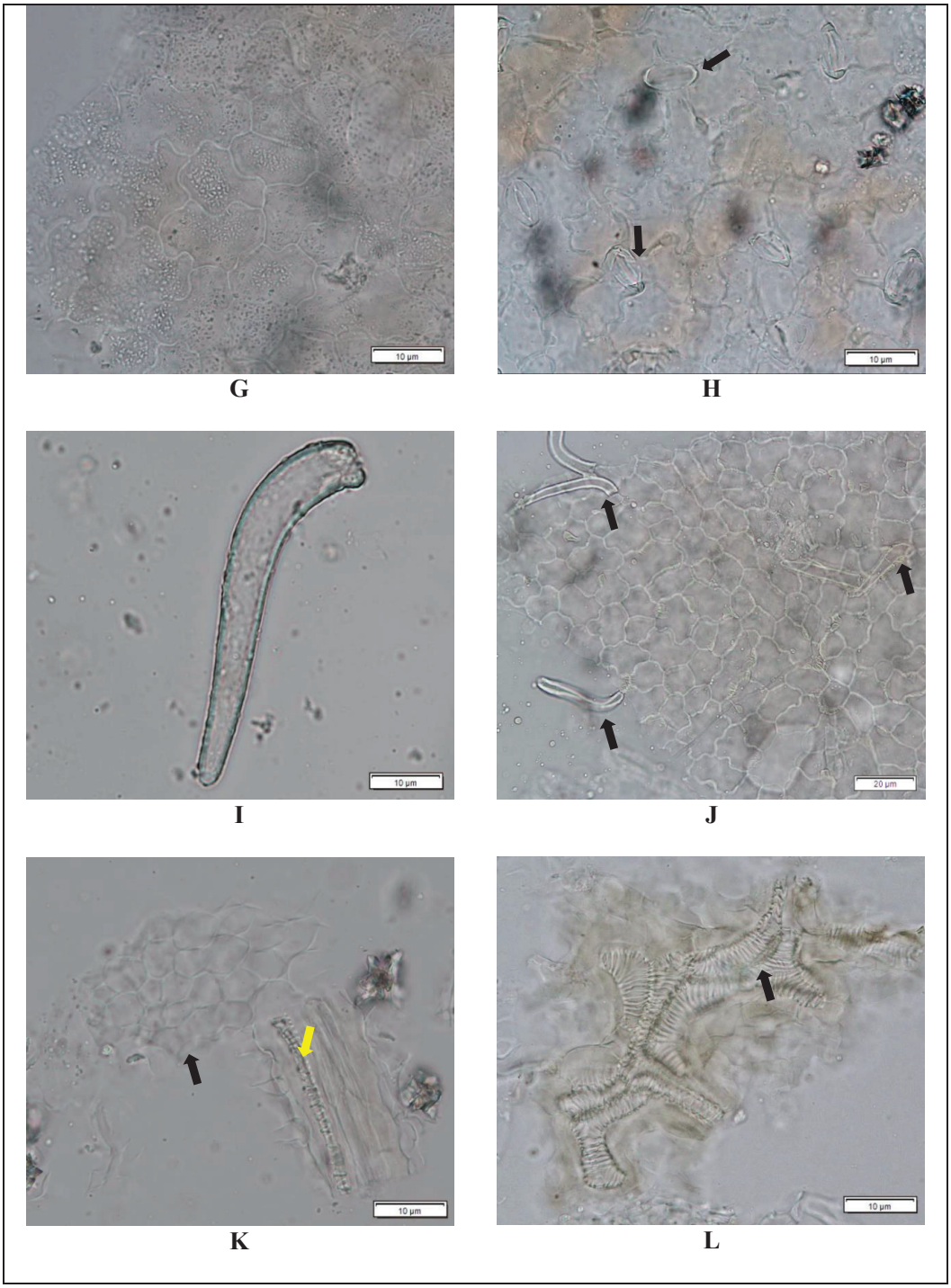


Figure 1 Plant microscopic identification of MO leaf extract (SBID 024/023) using powdered microscopy. (A) Starch grains viewed between crossed polarizes, hilum at the centre (arrow) (magnification 40×), (B) starch grains (magnification 40×), (C) idioblast cells (arrow) (magnification 40×), (D) calcium oxalate crystal in idioblast cells (arrow) (magnification 40×), (E) calcium oxalate crystal (druses) (arrow) (magnification 40×), (F) oil globules observed with Sudan red (III) (magnification 40×), (G) adaxial epidermis cells with sinous anticlinal walls (magnification 40×), (H) abaxial epidermis cells with sinous anticlinal walls, anomocytic stomata present (arrow) (magnification 40×), (I) simple, unicellular trichome (magnification 40×), (J) trichome cells on the epidermis cells (arrow) (magnification 40×), (K) parenchyma scalariform vessels (arrow) (magnification 40×) and (L) scalariform vessels (arrow) (magnification 40×)

Antioxidant activity of MO leaf extract after *in vitro* digestion

The effect of *in vitro* gastrointestinal digestion on the antioxidant activity is presented in Table 3. The result showed that all undigested (MO leaf extract) showed the highest activity in each assay. Among all assays tested, only the ABTS assay showed no significant difference ($p > 0.05$) between extract (undigested) and oral stage for both phases. The antioxidant activities revealed no obvious change after oral digestion, then the activity showed a downward trend in digestion process during intestinal stage. Overall, the current study exhibited that the antioxidant activities of MO leaf extract decreased (undigested > oral > gastric > intestine) after *in vitro* digestion, this includes in ABTS, ORAC, SOD and CUPRAC test. There was no significant difference between oral and gastric stages in the single phase (SOD assay) and between gastric and intestinal stages in the multiple phase (CUPRAC assay) ($p > 0.05$).

The opposite behaviour was observed in comparison with the previous assays and the DPPH assay, as activity increased after gastrointestinal digestion process. In the DPPH test, the results revealed that the percentage of radical scavenging was higher in the intestinal stage for both single and multiple phases compared to the oral and gastric stages ($p < 0.05$). Meanwhile, in the FRAP assay both single and multiple phases revealed high activity at the gastric and intestinal stages ($p > 0.05$).

The TPC test exhibited the highest phenolic content in the oral stage for both phases, followed by the intestinal and gastric stages ($p < 0.05$). As for the TFC test, the changes in antioxidant activity were similar to the changes in TPC in both phases. However, there was no significant difference between all stages in the TFC test. Generally, an overall reduction in the TPC and TFC of MO was discovered in the in vitro gastrointestinal digestion towards the end.

In terms of differences between single and multiple phases, the antioxidant activities shown from all assays tested the same trend and behaviour for digestion process. However, the antioxidant activities in the multiple phase (continuous digestion) were slightly lower for all tests. Statistical analysis was used to test between two phases (single and multiple), and the result showed there was a significant difference between those two phases in all stages and assays except for CUPRAC, TFC assay and ABTS (between intestinal stages).

Table 3 Pre- and post-digestion antioxidant evaluation of MO leaf extract

Sample	DPPH (%)	ABTS (%)	TPC (mg GAE/ 100 g)	TFC (mg QTN/ 100 g)	FRAP (mg TE/100 g)	ORAC (µmol TE/100 g)	SOD (%)	CUPRAC (mg TE/ 100 g)
Undigested	95.46 ± 0.001	59.96 ± 1.47^{a}	160900 ± 18	6394.88 ± 1081.74	508.93 ± 15.40	212000 ± 2500	89.53 ± 0.61	19.11 ± 7.27
Post-digestion (Single phase)								
Oral	15.57 ± 0.028^{a}	50.59 ± 1.81^{a}	59.0 ± 0.2^{a}	14.87 ± 2.31^{a}	95.82 ± 10.09^{b}	95000 ± 100^{a}	60.73 ± 1.01^{a}	14.10 ± 6.08 ^{a}
Gastric	16.03 ± 0.019^{b}	47.16 ± 11.77^{b}	26.0 ± 0.1^{b}	7.48 ± 1.48 ^{a}	233.44 ± 21.12^{a}	83000 ± 2200^{a}	65.48 ± 3.60 ^{a}	11.65 ± 0.31^{a}
Intestinal	50.62 ± 0.011^{c}	30.62 ± 1.13^{ab}	33.0 ± 0.5^{c}	6.14 ± 1.70 ^{a}	216.43 ± 9.55 ^{a}	48000 ± 6700^{b}	32.14 ± 0.80 ^{b}	11.88 ± 0.15 ^{a}
Post-digestion (Multiple phase)								
Oral	15.57 ± 0.028^{a}	50.59 ± 1.81^{a}	59.0 ± 0.2^{a}	14.87 ± 2.31^{a}	95.82 ± 10.09 ^{b}	95000 ± 100^{a}	60.73 ± 1.01^{a}	14.10 ± 6.08 ^{a}
Gastric	13.79 ± 0.007^{b}	11.06 ± 0.71^{b}	21.0 ± 0.0^{b}	9.28 ± 0.97 ^{a}	153.48 ± 21.81^{a}	38000 ± 870^{a}	29.55 ± 1.88^{b}	10.20 ± 0.21^{a}
Intestinal	27.65 ± 0.021^{c}	8.71 ± 1.17^{b}	31.0 ± 0.3^{c}	9.83 ± 4.30 ^{a}	112.07 ± 8.46 ^{a}	16000 ± 1400^{a}	21.93 ± 2.43 ^{b}	11.36 ± 0.33 ^{a}

Abbreviation- DPPH: 2,2-Diphenyl-1-picrylhydrazyl free radical scavenging, ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging, TPC: total phenolic compound, FRAP: Ferric reducing ability of plasma, ORAC: Oxygen radical absorbance capacity, SOD: superoxide dismutase, CUPRAC: Cupric reducing antioxidant capacity

Different superscript letters indicate significant difference within column and phases ($p < 0.05$)

Enzyme activity during *in vitro* digestion of MO

Table 4 shows the enzyme activities for each stage (separately used) in the *in vitro* digestion using MO. In the oral stage, high specific activity by α-amylase was revealed followed by pepsin (gastric) and trypsin (intestinal). The enzyme activity is directly proportional to enzyme concentration for all stages and phases. There was a significant difference between gastric (single phase) and gastric (multiple phase) in the specific activity, not the intestine stage.

Table 4 Enzyme activities in all *in vitro* digestion stages tested using MO leaf extract

Sample	Enzyme	Enzyme concentration (µg/mL)		Specific activity (U/mg)	
		Single	Multiple	Single	Multiple
Oral	Amylase	637.19 ± 5.94		38018.31 ± 354.65	
Gastric	Pepsin	64.66 ± 4.34^{a}	12.04 ± 0.77^{b}	1023.77 ± 1.13^{a}	952.91 ± 5.83^{b}
Intestine	Trypsin	746 ± 6.14^{a}	752.18 ± 10.21^{a}	0.29 ± 0.25^{a}	0.41 ± 0.30^{a}

Different superscript letters indicate significant difference within rows ($p < 0.05$)

Effect of *in vitro* digestion on antioxidant bioaccessibility index and residual antioxidant activity of MO leaf extract

The result for gastrointestinal digestion on the bioaccessibility index and residual antioxidant activity is presented in Table 5 and 6. A significant reduction was discovered (from extract to intestinal stage) in several assays, including the ORAC, ABTS, and SOD tests. The highest percentage of residual antioxidant activity was determined though the ABTS test, especially in the single phase method. No significant difference was observed in the residual antioxidant activity for FRAP and CUPRAC and bioaccessibility index for TPC and TFC, and it fluctuated throughout all digestion phases. However, the DPPH assay showed significant ($p < 0.05$) increase in both phase methods.

Table 5 Antioxidant bioaccessibility index of bioactive compound in MO leaf extract based on the antioxidant assay tested

Assay	Bioaccessibility index (%)				
	Oral	Single		Multiple	
		Gastric	Intestinal	Gastric	Intestinal
TPC	0.037 ± 0.20^{a}	0.016 ± 0.10^{a}	0.021 ± 0.50^{a}	0.013 ± 0.00^{a}	0.019 ± 0.30^{a}
TFC	0.26 ± 0.070^{a}	0.13 ± 0.32^{a}	0.11 ± 0.053^{a}	0.16 ± 0.050^{a}	0.15 ± 0.090^{a}
SOD	67.81 ± 0.66^{a}	73.12 ± 3.79^{b}	35.90 ± 0.80^{c}	33.00 ± 1.99^{b}	24.47 ± 2.64^{b}

Different superscript letters indicate significant difference within rows and phases ($p < 0.05$)

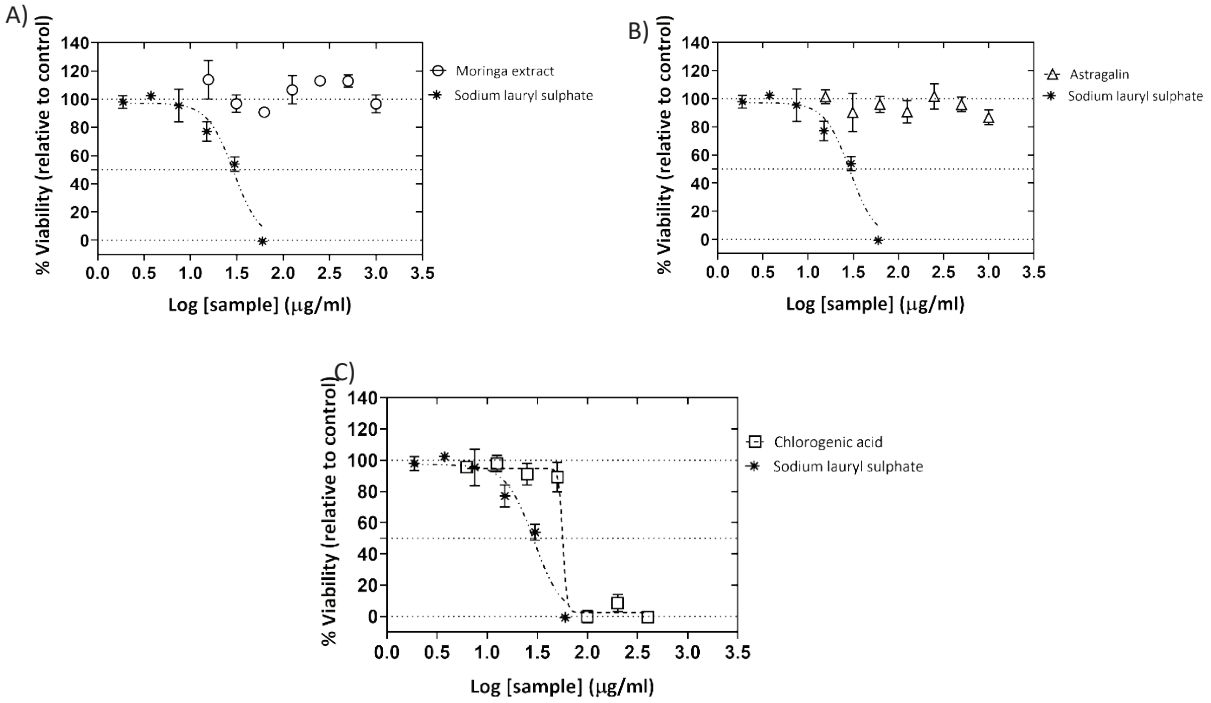
Table 6 Residual antioxidant activity of bioactive compound in MO leaf extract based on the antioxidant assay tested

Assay	Residual antioxidant activity (%)				
	Oral	Single		Multiple	
		Gastric	Intestinal	Gastric	Intestinal
DPPH	16.32 ± 2.05^{a}	16.79 ± 3.63^{a}	53.03 ± 2.04^{b}	14.44 ± 1.28^{a}	28.96 ± 4.02^{b}
ABTS	84.52 ± 3.98^{a}	79.30 ± 20.58^{a}	51.20 ± 3.00^{b}	18.45 ± 1.07^{b}	14.51 ± 1.80^{b}
FRAP	18.74 ± 1.48^{a}	46.21 ± 5.66^{b}	42.59 ± 2.15^{b}	30.40 ± 4.92^{a}	22.16 ± 2.38^{a}
ORAC	44.60 ± 0.76^{a}	39.37 ± 1.65^{a}	22.54 ± 3.34^{b}	17.80 ± 0.26^{b}	7.54 ± 0.38^{c}
CUPRAC	14.10 ± 6.08^{a}	11.65 ± 0.31^{a}	22.88 ± 0.14^{a}	10.20 ± 0.21^{a}	11.37 ± 0.33^{a}

Different superscript letters indicate significant difference within rows and phases ($p < 0.05$)

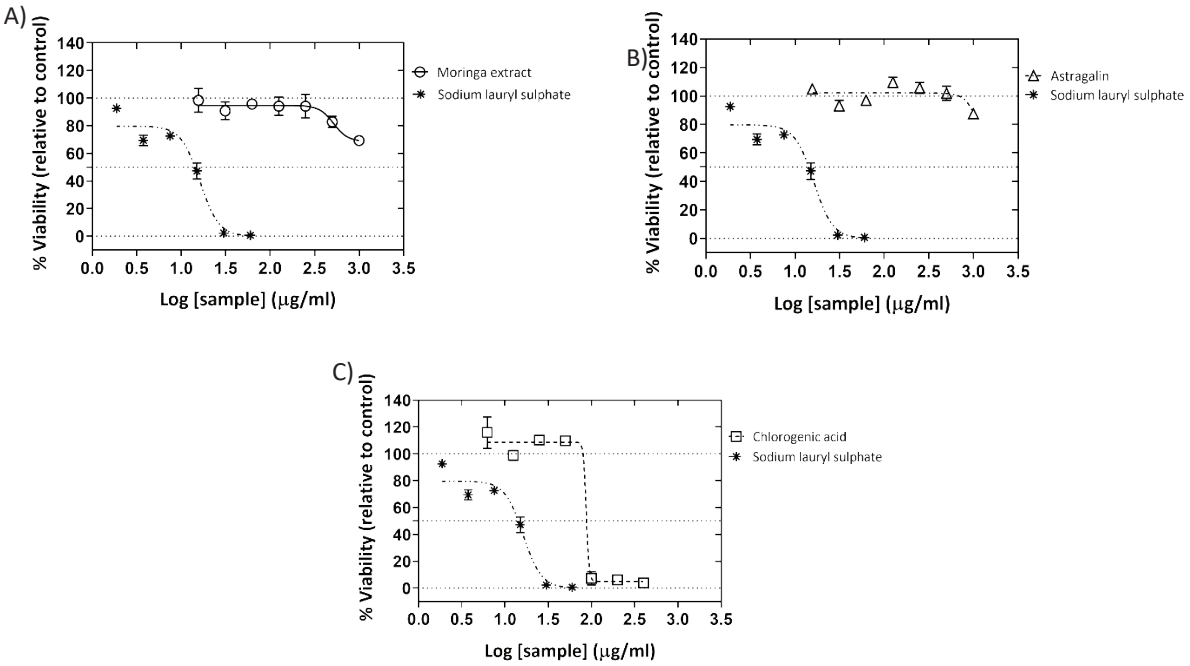
Cytotoxicity test of MO leaf extract and selected bioactive compounds

Vero and WRL-68 were used as in vitro kidney and liver cell models, respectively. Figure 2 shows the dose-response curves of MO leaf extract, astragalin and chlorogenic acid after 24 h of exposure on Vero cells while Figure 3 shows the effect on WRL-68 cells. MO leaf extract and astragalin did not affect the viability of Vero cells negatively after 24 h of exposure (Figure 2A and 2B). There is a slight decreased in viability of WRL-68 cells at the highest concentration tested (1000 µg/mL) when treated with MO leaf extract and astragalin (Figure 3A and 3B) but it is not very apparent. On the contrary, chlorogenic acid decreased both Vero and WRL-68 cells in a dose-dependent manner (Figure 2C and 3C) but to a lesser extent compared to sodium lauryl sulphate, the positive control. The 50% inhibition (IC50) values are tabulated in Figure 2 and 3 sodium lauryl sulphate exhibited the lowest IC50 values (Vero: 27.42 ± 3.10 µg/mL; WRL-68: 16.00 ± 0.99 µg/mL), followed by chlorogenic acid (Vero: 68.10 ± 3.75 µg/mL; WRL-68: 87.66 ± 9.65 µg/mL). The IC50 values for both MO leaf extract and astragalin were > 1000 µg/mL for both cell lines.



Sample/compound	IC_{50} (µg/mL)
MO leaf extract	>1000
Astragalin	>1000
Chlorogenic acid	68.10 ± 3.75
Sodium lauryl sulphate	27.42 ± 3.10

Figure 2 Dose-response curves of (A) MO leaf extract, (B) astragalin and (C) chlorogenic acid on Vero cell line after 24 h of exposure. Data are shown as mean SEM (n ≥ 3 independent experiments)



Sample/compound	IC_{50} (µg/mL)
MO leaf extract	>1000
Astragalin	>1000
Chlorogenic acid	87.66 ± 9.65
Sodium lauryl sulphate	16.00 ± 0.99

Figure 3 Dose-response curves of (A) MO leaf extract, (B) astragalin and (C) chlorogenic acid on WRL-68 cell line after 24 h of exposure. Data are shown as mean SEM (n ≥ 3 independent experiments)

DISCUSSION

Phytochemicals are bioactive compounds produced by plants in their primary or secondary metabolism to protect them from external threats. Tannins, flavonoids and steroids are known to have important properties and can modulate metabolic processes. Predominantly, tannins have been used as active compounds in various applications such as medicine and beverages due to their antioxidant properties. Also, flavonoid possesses an important role in protecting plants from UV radiation by acting as free radical scavengers, including chelating metals and reactive oxygen species (ROS) (Roy et al. 2022). While, plant sterols, the subgroup of the steroid suggest that they can act chemically as antioxidants, modest radical scavengers and physically as membrane stabilisers (Vezza et al. 2022).

According to the guidelines from ASEAN Annex III (ASEAN 2017), a microbial enumeration test using specified microorganisms on the MO revealed an acceptable level of TAMC and TYMC for several routes of administration. In this study, both TAMC and TYMC were microbiology-safe for oral use. They met the criteria for microbiology quality, specific for the development of product 1) containing herbal drugs prepared through infusion and decoctions using boiling water (NMT 5×10^7 for TAMC and 5×10^5 for TYMC)

and 2) containing herbal drugs of extracts with demonstration methods required, such as extraction with low- strength water or ethanol at low temperature (not boiling) (NMT 5×10^5 for TAMC and 5×10^4 for TYMC). From microbial analysis, MO leaf extract contained a low concentration of *E. coli* bacteria. According to the ASEAN guidelines, NMT 10^3 CFU of the bacteria in 1 g or mL is allowed for sample infusion or decoctions in boiling water. MO leaves can be contaminated with bacteria, especially *E. coli* that comes from the environment (animal, manure, soil and water). Other microbes can also contaminate the leaves of the plant, including *Campylobacter, Salmonella, Bacillus* and *Cronobacterium* (Walia et al. 2019).

Trace elements are critical for healthy plant growth; however, they can be toxic to other organisms at a certain limit. The trace element toxicity of MO leaves collected from Ethiopia was found to be below the detection limit, specifically for Pb and Cd (Adefa & Tefera 2020). Meanwhile, Hg and As were detected in different types of leaf powder MO sample from Thailand, ranging from 0.03 to 0.24 mg/kg of metals (Limmatvapirat et al. 2015). Generally, heavy metal can occur naturally in all plants, and they could also be from the anthropogenic pollution (Tangahu et al. 2011).

The microscopic profile obtained in this study showed most of the characteristics of MO from the monogeneric family Moringaceae. For example, Moringa leaves simplicia powder fragment from Beringharjo Market Yogyakarta was found to have a rosette crystal of calcium oxalate, stomata and mesophyll (Fatmawati et al. 2021). This study also confirmed the microscopic parameter identified with Indonesian Herbal Pharmacopoeia Edition II on Moringa leaves. Similarly, the vertical section of leaf and leaf peel showed the same morphology of MO leaves (collected from Tamil Nadu, India), including the palisade layer, unicellular hairs and starch grains (Singh et al. 2020).

In vitro gastrointestinal digestion

Moringa oleifera leaf extract is a good source of antioxidant compounds, and their bioactivity can benefit human health after biological processes, including digestion. To ensure the availability of the compound, *in vitro* digestion study was performed to determine the antioxidant bioaccessibility of the phytochemical content and residual antioxidant activity during digestion process. The antioxidant properties of plant extracts are mostly associated with their phenolic contents. Nonetheless, it may be affected by the chemical transformation caused by various mechanisms during the digestion process. Hence, to determine the effect of *in vitro* gastrointestinal digestion on the antioxidant potential of MO, several antioxidant assays were performed.

Moringa oleifera do have high antioxidant activities and it was proven by past research using different types of established assays (Abdulkadir et al. 2015, Xu et al. 2019, Hadi et al. 2022, Peñalver et al. 2022). The high activity in the ABTS assay can be due to the availability of bioactive compound such as flavonoids, hydroxycinnamic derivatives and polyphenols since these compounds was found to be active towards $ABTS^{\cdot+}$ (Nenadis et al. 2004). Furthermore, saliva can enhance the polyphenol adherence to oral surfaces and allow the contribution of the redox status of the oral cavity (Wojtunik-Kulesza et al. 2020). For example, salivary mucins may cause the digestibility of specific polyphenols like quercetins, and able to give an antioxidant effect such as enhanced formation of nitric oxide and reduction of nitrous acid to nitric oxide (Takahama et al. 2007). Quercetin is one of the compounds that can be found in the MO leaf extract (Manguro & Lemmen 2007, Vergara-Jimenez et al. 2017). Similar studies were reported on gastrointestinal study using different samples; where the antioxidant activities decreased at the end of the gastrointestinal digestion process (Huang et al. 2021, Xiang et al. 2021, Mercatante et al. 2022). The changes in pH after the digestion process, from pH 3 in stomach to pH 7 in intestine stage, may lead to the substantial changes in the structure and physicochemical properties of the bioactive compounds. At the same time, different interactions may occur, causing oxidation and precipitation of phenolics in the presence of enzymes in the digestive mixture and other components such as polysaccharides (Ydjedd et al. 2017). These studies confirm that digestion can result in the release of bioactive compounds from their conjugated forms,

promoting their absorption in the upper part of the gastrointestinal tract or that the compounds undergo structural modifications such as hydroxylation, glycosylation, methylation and conjugation.

Previous study has proven similar outcomes in DDPH and FRAP assays on the *in vitro* digestion study using Adzuki beans as their sample (Li et al. 2022). High antioxidant activity was observed in the undigested stage, followed by the intestinal stage (approximately 3.5 mg TE/g and 2.5 mg TE/g in DPPH and FRAP, respectively). Furthermore, the same condition was exhibited through FRAP, DPPH and ABTS tests of the 'wedang uwuh' beverage (Fauziah et al. 2023). The highest antioxidant activities in the intestinal phase could be due to the highest phenolic and flavonoids amounts released from the digestion process in this stage. The plant extract after oral digestion may have had the lowest OH- free radicals scavenging ability, while increasing gradually in gastric and intestine digestion. The increase of the identified phenolic compound may account for the intestine's increased antioxidant activity during gastrointestinal digestion. The condition of the digestion process at an acidic pH may cause some phenolics to exhibit antioxidant activities, whereas at a neutral pH, others have displayed pro-oxidant activities (Ydjedd et al. 2017). Phenolic compounds are categorised as a large and diverse group of phytochemicals that induce many different classes of bioactive substances. In this regard, the difference between all antioxidant assays used in this study may not be due to the phenolic and flavonoids content, but rather to the possibility of polyphenol diversity present. Plus, many research studies have shown the peptides with lower molecular weights exert greater antioxidant activity than large polypeptides or original proteins (Ren et al. 2008, Zhu et al. 2008).

Food matrices usually have a gradual decrease as they pass through the digestive system (Ng & See 2019), nonetheless, a study reported a slight increase at the end of digestion (Komiloglu et al. 2015). A similar condition was observed in the current study, where the TPC result was slightly higher in the intestinal stage compared to gastric stage. This may be due to the high acidity of the stomach environment, which has a strong effect on the released of phenolic compounds (Ng & See 2019). In comparison to the TPC test, the TFC result exhibited low antioxidant activity in this study due to fact that flavonoids are part of the plant extract's total phenolic content. Using Stinging Nettle and *Torilis leptophylla* L in past research, similar outcomes were found in both assays tested (Saeed et al. 2012, Fattahi et al. 2014). Notably, the TPC and TFC of digested samples may be undervalued since the methods employed only determine the soluble phenolic compounds, neglecting the presence of condensed phenolic compounds that are not recovered in the aqueous digesta media. This may include the proanthocyanidins compound and other insoluble polymeric phenolic compounds (Pinto et al. 2023).

Each stage of the digestive process (salivary, gastric, and intestinal) is a complex process that involves the presence of enzymes such as carbohydrate-hydrolyzing enzymes, lipases, proteases and bile salts (Martinez-Gonzales et al. 2017). There is a strong association between *in vitro* digestion and enzyme activity, where this method can further analyses specific enzymes to obtain maximum digestibility values or initial rate hydrolysis (Santos et al. 2019).

The low antioxidant bioaccessibility index and residual antioxidant activity of bioactive compounds after digestion may be caused by the interactions existing in the samples or food matrix and/or the way in which they were converted, which can lead to the low solubility of the sample in gastrointestinal fluids. This phenomenon suggested that the bioactive compound was less efficiently released after a chemical reaction, and this might be due to the low contact time with the enzymes. The dissimilarities in different assays of the samples resulted in different bioavailability values of the total quantity of compounds to be absorbed at the end of digestion. The reduction after gastric digestion to intestinal digestion could also be caused by the sensitivity of the bioactive compound to the pH changes, the availability of proteolytic enzymes or the formation of other complex constituents (such as bile salt). Thus, it can be assumed that the bioactive compound possibly cooperates with the dietary constituents during the digestion process and affects the bioavailability of food components (Mihaylova et al. 2021). A similar study

reported on the bioaccessibility of antioxidants using biofortified cowpea cultivars BRS Arace and Tumucumaque in FRAP and ABTS assays (Barros et al. 2021). Both FRAP and ABTS assays showed a significant decrease in antioxidant activity from the duodenal to colonic phase, indicating either low extraction of the food matrix or reduced metabolism. Some phenolic compounds that ester linked to the cell wall cannot be extracted through solvent extraction, and they require chemical hydrolysis to completely dissociate. This can be resolved by enzyme secreted from colonic bacteria, which can promote the hydrolysis of carbohydrates and release phenolic compounds during digestion and fermentation in the intestines.

In vitro cytotoxicity

Cytotoxicity tests are a rapid method to assess a chemical compound's effects on a human cell line (Bacskay et al. 2017). This helps in determining the potential toxic effect of compounds to human health that may occur during use. Despite the many potential benefits of MO, further research is needed to fully understand its effects, including cytotoxicity studies and the establishment of recommended dosages. Large quantities of MO, particularly the leaves or powder, might cause digestive issues such as diarrhea or stomach aches (Mackelden & Jampolis 2023). If the plant extract is found to be toxic to liver or kidney cells, it suggests that its systemic distribution might be problematic. This potential issue could also be reflected in its permeability across the intestinal barrier during in vitro digestion studies using the Caco-2 cell culture model. Thus, cytotoxicity testing on cells is one method that can be used to ascertain the safety level of the plant extract prior to its introduction to human cells for subsequent applications, such as in the food and pharmaceutical industries.

In this study, cytotoxicity test was used to measure the ability of MO leaf extract, astragalin and chlorogenic acid to cause cell damage or death. Generally, astragalin and chlorogenic acid are the significant phytochemical present in the MO leaves which are attributed to the antioxidant activities (Verma et al. 2009).

Moringa oleifera leaf extract exhibited non-cytotoxic activity at all tested concentrations in Vero cells, but weak cytotoxicity was observed at the maximum concentration of 1000 µg/mL in WRL-68 cells. However, astragalin did not demonstrate any cytotoxic effects in either Vero or WRL-68 cells in a dose-dependent manner. The IC_{50} values for both cells and test samples (MO and astragalin) were higher, indicating lower toxicity of the test samples.

In general, percentages of cell viability above 80% are considered non-cytotoxic, between 80-60% as weak, 60–40% as moderate, and below 40% as strong cytotoxicity (Lopez-Garcia et al. 2014). A similar study reported the cytotoxicity of MO leaf extract using concentrations of 125, 250, and 500 µg/mL on the normal Vero cell line. The IC_{50} result was found to be greater than 100 µg/mL after 96 hours, confirming the safety of the plant (Abbas et al. 2015).

Another study also indicated that astragalin compound had no toxicity or effects on proliferation in a normal colon mucosal epithelial cell line, consistent with our study but conducted on different cell lines (Yang et al. 2021). This study demonstrated that exposure to astragalin (0 to 80 µg/mL) had no significant effect on the cell line's viability over 24 to 72 hours, with 100% cell viability maintained. Thus, astragalin may be considered non-cytotoxic to most normal human cell lines at certain concentrations and exposure times.

Chlorogenic acid typically exhibits low cytotoxic effects on various normal cell lines. This is attributed to its ability to inhibit the growth of cytotoxic cells such as breast cancer and liver cancer cells, while maintaining high cell viability percentages in normal cells (Song et al. 2018, Zahra et al. 2020). In these studies, chlorogenic acid was used at low concentrations ranging from 70 to 170 µg/mL, similar to the concentration employed in the current study, where high viability of Vero and WRL-68 cells was observed at low concentration of chlorogenic acid (0 to 50 µg/mL). At these concentrations, the cell viability percentage exceeded 90%, with IC50 values higher than the positive control (sodium lauryl sulphate) and can still considered safe. However, the effective and therapeutic dose of chlorogenic acid should be approached with caution.

Typically, chlorogenic acid has low cytotoxicity effects on the different normal

cells line. This is because it able to inhibit the growth of cytotoxic cells such as breast cancer and liver cancer cells, and have high cell viability percentage in the normal cell (Song et al. 2018, Zahra et al. 2020). The concentration of chlorogenic acid used in these studies were at low concentration (70 to 170 µg/mL), which similar to the concentration used in the current study, where high viability of Vero and WRL-68 cells showed at 0 to 50 µg/mL of chlorogenic acid. At this concentration, the cell viability percentage was more than 90% as IC_{50} values higher than positive control. The effective and therapeutic dose of chlorogenic acid should be exercised with caution but can still be considered to be safe compared to sodium lauryl sulphate.

CONCLUSION

This study concluded that phenolic and antioxidant compounds in MO were sensitive to the gastrointestinal environment. The *in vitro* gastrointestinal digestion of MO significantly influences the bioaccessibility of bioactive compounds, as well as the residual antioxidant activity. The undigested MO, which is the original plant leaf extract, has exhibited incredible antioxidant activity compared to the activity in digested samples. Optimising factors that affect the bioaccessibility of bioactive compounds—including their release from the food matrix, particle size, and the various pH-dependent transformations during degradation, hydrolysis, and oxidation in the gastrointestinal tract, can enhance their absorption and efficacy. This improvement in bioaccessibility allows for better systemic circulation of MO, potentially leading to more effective therapeutic outcomes in clinical applications. Additionally, understanding how MO's bioactive compounds are metabolised by human gut microbiota, as well as their impact on and absorption by human primary cells, is essential. Future studies should focus on these aspects. Wheares, the IC_{50} value for MO indicated no or less cytotoxic effects on both cell line used in this study. The permeability test for the plant extract and antioxidant compound can be further explored to determine the potential bioavailability of bioactive compounds through *in vitro* cell culture models.

ACKNOWLEDGEMENTS

This work was funded by the research grant under RMK-12: Penerokaan Potensi dan Pembangunan Entiti Bernilai Ekonomi daripada Hasilan Semula Jadi ke Arah Memakmur Sektor Bioekonomi dan Kemampanan Produktiviti Biodiversiti Negara (24010701001). Special thanks to Natural Products Division, Forest Research Institute Malaysia (FRIM) for their expertise and support in facilitating this research.

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