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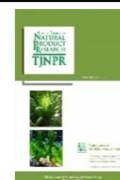

Antioxidant, Lipase Inhibitory Potential, and UHPLC Profile of Different Fractions of *Moringa oleifera* Leaves

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ABSTRACT

Moringa oleifera leaves have been consumed as vegetables in several regions of Indonesia. *M. oleifera* leaves contain many bioactive compounds that benefit human health. This study aimed to investigate the potential of *M. oleifera* leaf extract as an anti-obesity pharmaceutical alternative via lipase inhibitory activities. Aside from that, we aim to study the effect of different solvents on the bioactivity of its compounds. In this study, *M. oleifera* leaf powder was extracted using ethanol 70% and the extract was concentrated in vacuo at 40 °C to obtain a dried crude extract. The dried crude extract was fractionated using hexane, chloroform, ethyl acetate, and water. The total phenolic (TPC), total flavonoid content (TFC), antioxidant activities, the UHPLC profile of the fractions, and their lipase inhibitory potential were evaluated using standard methods. The results showed that the TPC ranged from 49 to 182.7 mg GAE/g, while the TFC ranged from 16.2 to 48.1 mg QE/g extract. The DPPH (1,1-Diphenyl-2-picrylhydrazyl) antioxidant activity ranges from 75.6 to 99.7 µmol TE/g extract, while the FRAP (Ferric ion reducing antioxidant power) activity ranges from 50.1 to 99.4 µmol TE/g extract. Moreover, the inhibition of pancreatic lipase activity varies from 58.1 to 74.3%, in contrast to orlistat which has a higher inhibition rate of 95.3%. UHPLC (Ultra-High-Performance Liquid Chromatography) indicated that the water fraction contained more compounds than others due to its dominant and highest peak. This study shows that *M. oleifera* leaves have the potential to be developed as a source of anti-obesity and antioxidant agents.

Keywords: Antioxidant, *Moringa oleifera*, Pancreatic lipase inhibitory, Total flavonoid content, Total phenolic content, UHPLC-PDA.

Introduction

Moringa oleifera, commonly known as drumstick tree, is recognised as a functional food due to its nutritional value and health benefits. *M. oleifera* is a species belonging to the Moringaceae family, a native plant of the Himalayan foothills of southern India, which now can be found in the tropical and subtropical regions of the world.[1,2] Several Asia and African countries use *M. oleifera* as a traditional medicine and food ingredient. In India, the plant is utilised in the preparation of curries, sambars, salads, and dals. In Africa, people use the leaves and flowers of *M. oleifera* to fortify soups and in weaning foods, as well as to enrich the protein content of yoghurt.[3] The leaves are also used in many sauces and other food preparations. Furthermore, it is consumed as traditional medicine in Burkina Faso.[4] Even though the drumstick tree is well known and can easily be found in Indonesia, the potential is still not fully utilised. Several regions have consumed the leaves as a vegetable because they are easy to maintain. The bark can be scraped down to the wood and then sprinkled on top of the meat or fish being boiled.[5] In Aceh, it is traditionally used as herbal medicine to treat seizures.[6] There are many studies on the use of this plant as a functional food, but it is not yet widely known by some local communities in Indonesia.[7–9] Furthermore, there is currently limited research on the lipase inhibitory activity of this plant and its possible utilisation as an anti-obesity alternative medicine.[10] *M. oleifera* has many health benefits, because every part of it, including the flowers, leaves, seeds, and pods, is edible and high in nutrients and bioactive components. Extracts of leaves, fruits, and seeds using various solvents, including ethanol, methanol, water, and ethyl acetate, have shown some activities such as hypolipidemic effects in hypercholesterolemic rats, as well as anti-inflammatory, antioxidant, and antimicrobial activity.[11,12] Moringa seed oil is used as a topical remedy for rheumatism and gout. The flower is also known for its aphrodisiac and anti-inflammatory properties, while the leaves are considered vegetables.[13,14] According to the literature review, the genus Moringa has been found to contain over 90 different compounds, including glucosinolates, flavonoids, isothiocyanates, terpenoids, alkaloids, and lignans. Therefore, the therapeutic properties of Moringa species may be associated with this wide range of chemical variations. *Moringa oleifera* leaf extracts have demonstrated efficacy as an antioxidant, antibacterial, and anticancer agent and also promoted erythrocyte production in pregnant women.[15–18] The primary flavonoids found in *M. oleifera* leaves are myricetin, quercetin, and kaempferol.[19] Among these, quercetin, iso-quercetin, and quercetin-3-O-malonylglucoside have been reported to possess antiobesity properties.[20–22] *M. oleifera* leaf extract exhibits anti-obesity effects via inhibiting lipase and cholesterol esterase, suggesting its potential in the prevention and treatment of hyperlipidemia.[23] Pancreatic lipase breaks down the triacylglycerols found in the gastrointestinal tract, making it the primary enzyme that breaks down and helps the absorption of lipids.

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The inhibition of pancreatic lipase plays a role in limiting intestinal fat absorption, thus controlling hyperlipidemia.[24] Other studies report that *M. oleifera* leaves contain bioactive compounds such as vitamins (retinol, *β*-carotenes, ascorbic acid, and tocopherols), saponins, tannins, and phytates.[25,26] In addition, other phenolic compounds such as gallic acid, chlorogenic acid, luteolin, rutin, apigenin, vanillin, omega fatty acids, *β*-sitosterol, and moringinine have also been identified in *M. oleifera* leaves.[27] In this study, the components of the crude extract were separated using solvents with different polarities. The objective of this study was to compare the total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (DPPH and FRAP assay), and lipase inhibitory activity of each fraction. In addition, this study also reports the chromatograms of the fractions using UHPLC to check the retention times and peaks of each fraction.

Materials and Methods

Sample Preparation
Moringa leaves were obtained from the collection and experimental garden of the Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI), Bogor Regency, West Java, with voucher number BMK0128092016. The samples were dried using a Fluidized Bed Dryer (FBD) for 3 hours at 40 to 60°C. FBD was utilised in this study because it improves the drying process by circulating hot air from below, ensuring more surface drying. The temperature was adjusted so that it was neither too low, which would take longer to dry, nor too hot, which would destroy the biochemical compounds. The dry leaves were ground and then sieved through a 40-mesh sieve to obtain fine Moringa powder. The dried powder was stored in an airtight container in the freezer at -18°C until further study.

Sample Preparation and Fractionation for Bioactivity Studies
A total of 10 g moringa leaf powder was immersed in a solution of 70% ethanol (1:2, w/v). The mixture was then subjected to ultrasonic waves (Bransonic Ultrasonic Cleaner 8510E MTH, USA) for a duration of 30 minutes at 20 to 25°C (room temperature). The mixture underwent filtration, and the residue was subjected to further treatment using ultrasonication, following the same procedure as previously described. The filtrate from the first and second extractions was collected and concentrated using a rotary evaporator (Buchi Rotavapor R-300, Buchi Labortechnik Switzerland) at a temperature of 45°C until a solid extract was achieved. The dry extract (crude extract) was fractionated using a liquid-liquid extraction method with four types of solvents (water (A), hexane (H), chloroform (K), and ethyl acetate (E)). The filtrates from the fractionation were then concentrated with a rotary evaporator at a temperature of 30°C. The crude extract and the fraction were used for the predetermined bioactivity studies.

Determination of Total Phenolic Content (TPC)
Initially, 10 mg of crude extract and fractions were dissolved in 10 mL ethanol (1000 µg/mL), then mixed with 100 µL of 1 N Folin Ciocalteu 10% (v/v) reagent. Afterwards, 800 µL of 700 mM sodium carbonate was added and incubated for 2 hours at 20 to 25°C. A total of 200 µL of the mixture was transferred to a 96-well microplate, and absorbance was measured at 750 nm (Epoch Microplate Spectrophotometer, BioTek® Instruments Inc. USA). The determination was carried out in triplicates, and gallic acid (concentration 20-200 µg/mL) was used as the standard. The total phenolic content was expressed in mg Gallic Acid Equivalent (GAE) per g dry extract.[28]

Determination of Total Flavonoid Content (TFC)
A 10 µL volume of crude extract or fractions (1000 µg/mL) was transferred to a microplate. Then, 60 µL of ethanol, 10 µL of $AlCl_3$ solution (10% w/v), 10 µL of CH_3COOK (1 M), and 120 µL of distilled were added sequentially. The solution was subjected to incubation for 30 minutes at 20 to 25°C. The absorbance value was measured at λ 415 nm (Epoch Microplate Spectrophotometer, BioTek® Instruments Inc. USA). A standard solution of quercetin ranging from 20 to 200 µg/mL was utilised. Total flavonoid measurement is reported in milligrams of Quercetin Equivalent (QE) per gram of dry extract.[29]

Antioxidant Activities
DPPH radical scavenging activity assay
DPPH antioxidant assay refers to the Salazar-Aranda[30] method, with slight modification. First, 100 µL of extract (crude extract and fractions at a concentration of 1000 µg/mL) was added to 100 µL DPPH solution (125 µM) in ethanol, then homogenised and incubated at 20 to 25°C in darkness for 30 minutes. The decrease in absorbance was measured using a microplate reader at a wavelength of 517 nm. This test was repeated three times, and the antioxidant capacity was expressed in Trolox Equivalent (TE) per g of dry extract (µmol TE/g dry extract).

FRAP assay
FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 1 mM of freshly prepared TPTZ solution (in 40 mM HCl), and 20 mM $FeCl_3$ solution (10:1:1 v/v/v). Trolox was used as a standard solution with 80-800 µmol/L ethanol concentration. The standard concentration range was chosen based on preliminary experiments to determine the concentration that provides the optimum absorbance value. As a result, this concentration may vary depending on the equipment used. A total of 20 µL of sample extract (crude extract and fractions at a concentration of 1000 µg/mL) were put into a 96-well microplate, followed by the addition of 180 µL of FRAP reagent. The mixture was incubated at 37°C for 15 minutes; then, absorbance was measured at 595 nm using a microplate reader. The FRAP result is reported as µmol Trolox Equivalent/ g extract.[31,32]

Pancreatic Lipase Inhibition Activity
Lipase inhibitory activity was measured using *p*-nitrophenyl butyrate (*p*-NPB) as substrate, based on the method of Chedda *et al.* [33] and Pliego *et al.*[19], with slight modification. First, 25 µL of the crude extract and fractions (concentration of 1000 µg/mL) were mixed with 100 µL of 0.1 mM phosphate buffer solution (PBS, 150 mM sodium chloride, and 0.5 % (v/v) Triton-X-100, pH 7.2), 50 µL enzyme lipase L3126 Sigma Aldrich, USA (concentration 300 µg/mL dissolved in 0.1 mM PBS pH 7.2), and 25 µL of *p*-NPB (concentration 0.4 M dissolved in acetonitrile). After 30 min incubation at 37°C, the absorbance was measured in a microplate reader at 415 nm. The test was repeated three times. Orlistat was used as a positive control at the same concentration as the extract. The determination of the chemical and other parameters used in this procedure was based on previous studies with similar samples or conditions to this study.[34] The inhibitory activity was expressed as percent inhibition and was calculated from the following equation:

$$\text{Inhibitory activity (\%)} = \frac{\text{(Absorbance of blank-Absorbance of sample)}}{\text{Absorbance of blank}} \times 100 \quad (1)$$

UHPLC Analysis
Sample extracts from four fractions (hexane, chloroform, ethyl acetate, and water) were diluted to a concentration of 50 ppm using acetonitrile HPLC grade. Each fraction was filtered using a nylon membrane filter with a size of 0.22 µm. Each fraction was analysed using the UHPLC instrument (Nexera LC-40B XR, Shimadzu, MD, USA) based on the method of Lin *et al.* [35] with slight modifications. A total of 5 µL of each extract was injected automatically into the LC system (SIL-40C XR Autosampler), and the UPLC@BEH column was 1.7 µm C18 (2.1 x 50 mm, Waters, MA, USA). The absorbance value was scanned using a PDA detector from 190 – 800 nm, but the absorbance was observed at 270 nm. The mobile phases comprised eluent A (0.1% formic acid in water, v/v) and eluent B (0.1% formic acid in acetonitrile, v/v) with a 0.4 mL/min flow rate. The elution conditions were: 0–2 min, 10% B; 2–26 min, 10–100% B. The temperatures of the column and autosampler were 30°C and 15°C, respectively.

Statistical analysis
SPSS® (Statistical Package for the Social Science) version 24.0 was used to analyse the data. one-way Analysis of Variance (ANOVA) was used to assess bioactivity data from 5 samples (4 fractions and crude extract) with a 95% confidence interval. The post-hoc test was carried out using Duncan's Multiple Range Test at a 5% level.

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Results and Discussions

Total phenolic and total flavonoid content
Moringa leaves are rich in various polyphenolic compounds such as flavonoids, ascorbic acid and carotenoids. However, the composition of the bioactive content of plants depends on several factors, including the physiological stage of the plant, climatic conditions, and geographical origin.[1,36] Besides that, the extraction method is crucial to differences in the bioactive composition of Moringa leaves. The total phenol and flavonoid content were determined using *in vitro* spectrophotometric assays (such as the Folin Ciocalteu and $AlCl_3$ methods). The results of total phenolic content (TPC) and total flavonoid content (TFC) from various *M. oleifera* extracts are shown in Figure 1. Different samples (crude extract and fractions) gave varied TPC based on the statistical analysis of the results. The post hoc test results showed that the water (aqueous) fraction had the highest total phenol content, 182.7 mg GAE/g extract. Furthermore, TPC from the crude extract and chloroform fraction did not differ significantly, with 92.8 and 91 mg GAE/g extract values, respectively. The TPC values of the ethyl acetate and hexane fractions were 70.1 and 49 mg GAE/g extract, respectively. A review by Leone *et al.* found variations in the phenolic content of *M. oleifera* from multiple investigations, ranging between 20.90 to 122 mg GAE/g dry weight, indicating that some samples have higher phenolic content than those obtained in this study, while others are lower.[26] Meanwhile, Ola-Adedoyin *et al.* found that *M. oleifera* leaf extract contains 0.5964 mg GAE/ g dry mass of phenolic content. [37] This value was far lower than in this study, which could be due to differences in extraction methods because Ola-Adedoyon *et al.* directly dissolved dried leaves in water used in their study without any prior extraction. This may influence the optimal extraction of the targeted compound. In contrast to TPC, the highest total flavonoid content (TFC) was observed in the crude extract with 48.1 mg QE/g extract, followed by the water fraction with 37.1 mg QE/g extract. Meanwhile, the total flavonoid content of the other three fractions (chloroform, ethyl acetate, and hexane), with values of 16.9 mg QE/g extract, 23.9 mg QE/g extract, and 16.2 mg QE/g extract, were not significantly different. These numbers are higher than the study by Leone *et al.*, which showed TFC ranging between 5.059 and 12.16 mg/g dry weight. [26] The differences in TPC and TFC between these studies might indicate that different origins, extraction methods, drying methods, or other factors may likely affect the total bioactive compound in the plant.

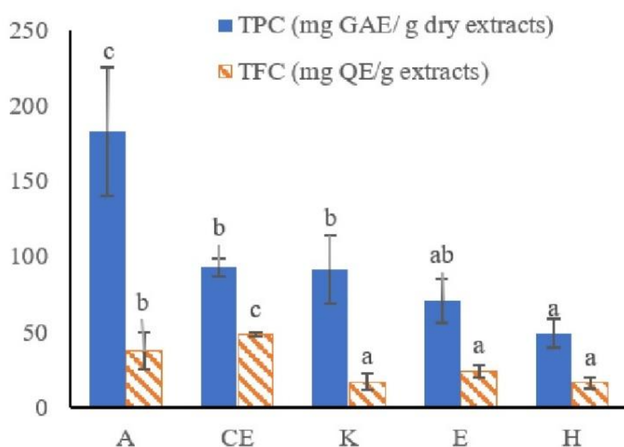

Figure 1: TPC and TFC of *M. oleifera* extract at a concentration of 1000 µg/mL; the results are shown as mean ± SD. A: water fraction; CE: crude extract; K: chloroform fraction; E: ethyl acetate fraction; H: hexane fraction. Means from similar bar colours with at least one same letter are not significantly different based on Duncan's Multiple Range Test ($p > 0.05$).

Antioxidant activities
Antioxidants have two mechanisms of action in neutralising free radicals in biological systems: single electron transfer (SET) and hydrogen atom transfer (HAT). Based on this mechanism, more than one test method can be used to determine the antioxidant activity of a plant extract.[38] Antioxidant activity in this study is expressed in mol Trolox Equivalent (TE)/g extract units. Antioxidant activity from *M. oleifera* extracts with two different test methods is presented in Table 1. The results showed that the water fraction had the highest DPPH radical scavenging activity with a value of 99.7 µmol TE/g extracts, followed by ethyl acetate fraction at 87.0 µmol TE/g extracts. In comparison, the DPPH radical scavenging activity of the other three fractions was not significantly different. On the other hand, the highest ferric-reducing antioxidant power (FRAP) was shown by hexane fraction (99.4 µmol TE/g extract), while the FRAP activity of the other fractions was not significantly different (p>0.05).

Table 1: Antioxidant activity of *M. oleifera* extracts

M. oleifera fractions	Antioxidant activity (µmol TE/g extract)	
	DPPH	FRAP
Water	99.7 ± 3.3^{b}	50.1 ± 3.6^{a}
Ethyl acetate	87.0 ± 12.2^{ab}	74.4 ± 16.7^{a}
Chloroform	76.9 ± 15.8^{a}	55.1 ± 14.1^{a}
Hexane	75.6 ± 17.3^{a}	99.4 ± 34.6^{b}
Crude extract	78.2 ± 1.8^{a}	50.9 ± 2.9^{a}

Note: Different superscript letters in the same column indicate statistical differences according to Duncan's multiple comparison test at p = 0.05. TE = trolox equivalent; DPPH = 1,1-diphenyl-2-picrylhydrazyl; FRAP = ferric-reducing antioxidant power

The hexane fraction in this study showed a greater FRAP activity than the DPPH free radicals scavenging activity, indicating differences in their mechanisms. In the DPPH assay, the scavenging action may be due to the hydrogen-donating ability. In contrast, the scavenging of FRAP is due to the scavenging of proton radicals induced by donating electrons.[39] The antioxidant activity of DPPH and FRAP from *M. oleifera* extracts depends on the solvent used in the extraction. The different compounds can be extracted with different solvents due to different solubility. Unlike our study, Fitriana *et al.* [40] reported the antioxidant activity of extracted Moringa leaves with several different solvents, including methanol, ethyl acetate, dichloromethane, and hexane. The results showed that the methanol extract had the highest antioxidant activity (DPPH and ABTS assay) with IC_{50} values of 49.30 µg/ml and 11.73 µg/ml, respectively. Methanol is the highest polar solvent among the other solvents, which can pull out more polyphenol compounds.[40]

Pancreatic lipase inhibition
Generally, dietary fat is degraded and digested with the help of pancreatic lipase, and the inhibitory activity of this enzyme can lower blood fat levels and improve blood lipid disorders. This study revealed that the chloroform and hexane fraction of *M. oleifera* exhibited promising pancreatic lipase inhibition values at 74.3% and 70.5%, respectively, compared with the positive control (orlistat) at 93.5 %, as shown in Figure 2. Both the crude extract and water fraction showed an insignificant difference in inhibiting pancreatic lipase activity, with 67.4 % and 62.9 % inhibition values. Ethyl acetate exhibited the lowest inhibition on pancreatic lipase among the other fractions, with a value of 58.1 %. A previous study reported that the ethyl acetate fraction of *M. oleifera* showed the highest lipase inhibitory activity, at 32.58% for the Cambodia sample and 20.91% for the South Korean sample, at a 0.1 mg/mL concentration.[21] This supported the hypothesis that different

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origins (soil, growing place, etc) may affect the bioactive compounds and the activity of the plant extract. Another study by Chen *et al.*[34] showed that the ethanolic extract of Moringa leaves exhibited promising inhibition of pancreatic lipase with an IC_{50} value of 181.3 µg/mL sample.

Chromatogram profiles by UHPLC-PDA

The chemical profiling of Moringa leaves was determined using UHPLC to examine various peaks of the detected components in the hexane, chloroform, ethyl acetate, and water fractions. Peaks of the chromatograms were detected using a PDA detector from λ 190 – 800 nm, while the chromatograms presented in this study were obtained from the absorption measurement at λ 270 nm (Figure 3). The wavelength of absorption selected in this study was 270 nm because it showed larger and more intense peaks for the four fractions of *M. oleifera* than other wavelengths. The chromatogram data provide a comprehensive perspective of the biological components that may have contributed to the fractions' bioactivity.

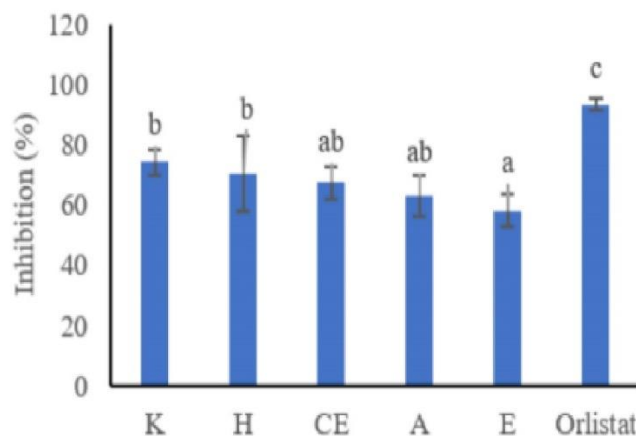

Figure 2: *In vitro* pancreatic lipase inhibitory effect of *M. oleifera* leaf extracts. A: water fraction; CE: crude extract; K: chloroform fraction; E: ethyl acetate fraction; H: hexane fraction. Means with at least one same letter are not significantly different based on Duncan's Multiple Range Test ($p > 0.05$).

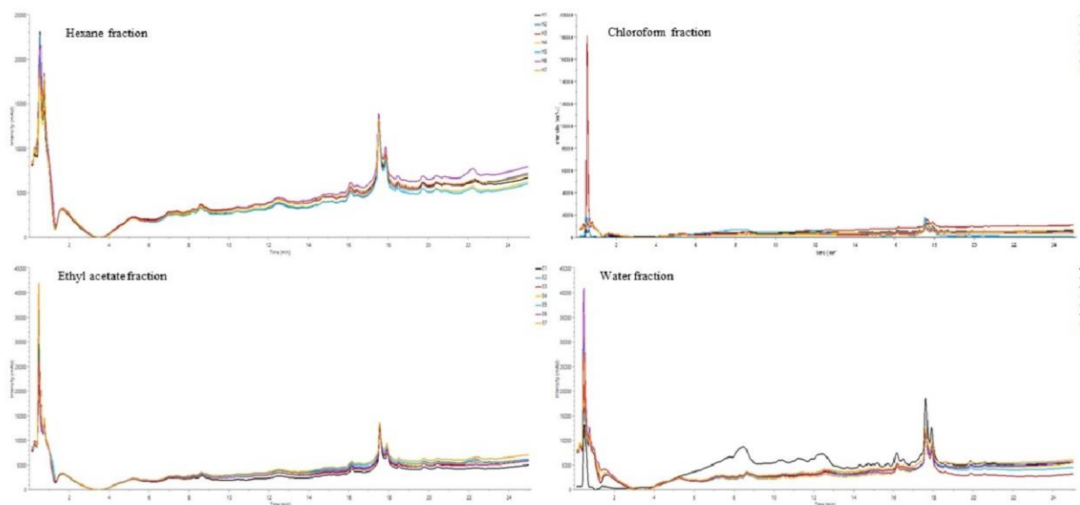

Figure 3: UHPLC-PDA chromatogram profile of *M. oleifera* fractions at λ 270 nm

Analysis of the chromatograms showed that water fraction exhibited the highest number of dominant peaks (8 peaks), followed by hexane (6 peaks), ethyl acetate (5 peaks), and chloroform fraction (3 peaks). All fractions showed similar chromatogram patterns at the retention time between the 17th and 18th minutes. The obtained retention time can be used as one of the markers to identify a compound.[41] However, to determine which components were present in four of the fractions, additional analysis utilising mass spectrometry was used in this study. Overall, the water fraction showed the most abundant and highest peak, indicating that it contained more compounds than other fractions. The peak of polar compounds could be detected first on the chromatogram and usually at shorter retention times, while non-polar compounds are strongly bound to the stationary phase so that peaks will emerge at longer retention times.[42]

Conclusion

The water (aqueous) fraction has the highest TPC (182.7 mg GAE/g extract) and antioxidant DPPH free radical scavenging activity (99.7 µmol TE/g extracts), while the crude extract has the highest TFC (48.1 mg QE/g extract). The hexane fraction showed the strongest FRAP antioxidant activity (99.4 µmol TE/g extract) compared to others. Furthermore, the chloroform and hexane fractions exhibited the strongest pancreatic lipase inhibitory potential (74.3% and 70.5%, respectively), with no significant difference between the two fractions. UHPLC-PDA analysis showed that all fractions have a similar pattern between the 17 - 18 minutes of retention time, but it also indicates that the water fraction contained more compounds than the others due to the dominant peak. Further research is required to identify the compounds in the samples using mass spectrometry to identify the components in the four *M. oleifera* fractions.

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Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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