https://doi.org/10.48047/AFJBS.6.16.2024.1841-1857

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Phenolic content and antioxidant activity of *Moringa oleifera* leaf extracts

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Volume 6, Issue 16, Dec 2024

Received: 15 Oct 2024

Accepted: 25 Nov 2024

Published: 16 Dec 2024

doi:10.48047/AFJBS.6.16.2024.1841-1857

Abstract

Moringa oleifera is a medicinal plant that is a source of nutrients. It is rich in protein, fatty acids, minerals, relatively high polyphenol compound, and has antioxidant activity. The present study investigates the extraction yield, phytochemical content, and antioxidant activity of *Moringa oleifera* leaves using two different extraction methods: maceration with methanol and aqueous decoction. The methanol extract showed a higher yield (11.82%) compared to the aqueous extract (8.35%). Phytochemical screening revealed the presence of polyphenols, tannins, flavonoids and quinones in both extracts. Quantitative analysis indicated that the methanol extract contained significantly higher total phenolic content (100.11±10.24 µg GAE/mg) and flavonoid content (52.85±0.46 µg QE/mg) than the aqueous extract (30.47±7.74 µg GAE/mg and 15.16±2.16 µg QE/mg, respectively). In HPLC investigation including 15 standard chemicals, we successfully identified chlorogenic acid in the *M. oleifera* aqueous extract. Furthermore, in the methanol extract, the main component was rutin. The antioxidant activity was assessed using DPPH radical scavenging assay, total antioxidant capacity (TAC), and ferric reducing antioxidant power (FRAP). The methanol extract exhibited a superior DPPH scavenging activity (IC50 = 70.24 µg/ml) compared to the aqueous extract (IC50 = 103.71 µg/ml), and higher TAC (50.42%) and FRAP (EC50 = 815.5 µg/ml). These findings highlight the influence of extraction methods on the phytochemical and antioxidant profiles of *M. oleifera* leaves, suggesting that methanol is more effective solvent than water for extracting bioactive compounds.

Key words: *Moringa oleifera,* Antioxidant activity, Polyphenols, HPLC-PDA.

1. Introduction

Medicinal plants, renowned for their rich bioactive components, play a crucial role in drug development and therapeutic applications (Asekunowo *et al.,* 2022). One notable example is *Moringa oleifera,* often referred to as 'Mother's Best Friend' or the 'Miracle Tree,' highlighting its exceptional medicinal properties and its significance in human health (Kalauni *et al.,* 2023). *Moringa oleifera* (*M. oleifera)* belongs to the kingdom Plantae, with the division Magnoliophyta, class Magnoliopsida, order Brassicales, family Moringaceae, and genus/species *M. oleifera* (Abdull Razis *et al.,* 2014). *M. oleifera* is a perennial, nutritional, and flowering plant that is grown worldwide in the tropics and subtropics of Asia and Africa (Shih *et al.,* 2011). It is widely cultivated in India, Pakistan, and Arabia, and it is now distributed in the Philippines, Cambodia, Central, North, South America, and Caribbean Islands (Anwar *et al.,* 2007). Each part of this plant, including the bark, roots, fruits, flowers, leaves, seeds, and gum, contains a wealth of proteins, vitamins, and minerals such as potassium, calcium, phosphorus, iron, folic acid, and β-carotene (Kumar and Sharma, 2023), and they are used in traditional medicine to treat diarrhea, hypertension, and various folk remedies in many countries (Anwar *et al.,* 2007). Meanwhile, Leaves boast higher levels of vitamin A than carrots, calcium surpassing that of milk, iron exceeding spinach, vitamin C outmatching oranges, and potassium surpassing bananas. Additionally, the protein quality of *Moringa* leaves compared with that of milk and eggs (Fahey, 2005). *Moringa* leaves act as a good source of natural antioxidants and thus enhance the shelf life of fat-containing foods due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics, and carotenoids (Anwar *et al.,* 2007).

Phenolic compounds are defined as natural metabolites arising biogenetically from either the shikimate/phenylpropanoid pathway (Vuolo *et al.,* 2019). They are secondary metabolites, ubiquitous in plants and plant-derived foods and beverages, exhibiting a wide diversity of structures (Cheynier, 2012), which are made of an aromatic ring containing one or more hydroxyl substituents (Tsao, 2010) such as polyphenols. Polyphenols were defined as water-soluble phenolic compounds having molecular weights between 500 and 3,000 Da (Bate-Smith, 1962). Its basic monomer is the phenolic ring, which is generally classified as phenolic acids and phenolic alcohols. Based on the phenolic ring, polyphenols can be classified into

several classes, the main ones being phenolic acids, flavonoids, stilbenes, phenolic alcohols, and lignans (Abbas *et al.,* 2017).

Turning our attention to a specific subgroup within the realm of polyphenols, it's essential to explore flavonoids. Flavonoids are a group of polyphenolic compounds that can widely be found in fruits and vegetables (Farkas *et al.,* 2004). They have a basic chemical structure of C6–C3–C6, consisting of two aromatic rings joined by a three-carbon chain (Tizazu and Bekele, 2024). As phytoalexins or antioxidants, flavonoids can scavenge reactive oxygen species (Boudoukha *et al*., 2018) and protect plants against damage from biotic and abiotic stresses, including UV irradiation, cold stress, pathogen infection, and insect feeding(Liu *et al.,*2021).

The abundance of antioxidants previously mentioned in *M. oleifera* emphasizes its remarkable ability to combat oxidative stress (Antioxidants are groups of substances able to prevent and delay the oxidation of easily oxidizable molecules and avoid free radicals' formation in living organisms). (Suntar, 2023), which is a pathological condition resulting from the disparity between the production of free radicals and the body's capability to counteract them. Oxidative stress induces apoptotic signaling that is consequent to ROS (reactive oxygen species) increases and/or antioxidant decreases, disruption of intracellular redox homeostasis, and irreversible oxidative modifications of lipid, protein, or DNA (Circu and Aw, 2010). This imbalance can damage cells and contribute to many diseases, including cancer, neurological disorders, atherosclerosis, hypertension, ischemia/perfusion, and diabetes (Birben et al., 2012). Therefore, antioxidants play an important role in inhibiting and scavenging free radicals, thus protecting humans against infections and degenerative diseases (Sreelatha and Padma, 2009; Boudoukha *et al*., 2019). Consequently, the purpose of the present study is to evalate the phenolic compounds of the *M. oleifera* plant by estimating its content of polyphenols and flavonoids, estimation of antioxidant activity using DPPH free radical test, measurement of total antioxidant capacity (TAC) and FRAP method.

2. Materials and Methods

2.1. Chemical Material

Chemicals used in this study are quercetin, aluminum trichloride ($AlCl_3$), butyl hydroxyl anisol (BHA), 2,2-diphenyl-1-picryl-hydrazyl (DPPH), Folin-Ciocalteu, gallic acid, sodium carbonate, ascorbic acid, sulfuric acid (H_2SO_4), sodium phosphate (Na_3PO_4), ammonium

molybdate and iron chloride ($FeCl_3$) were used for antioxidant assays and supplied from chemical company Sigma-Aldrich (Germany).

2.2. Plant Material

Moringa oleifera dried leaves were procured from an herbalist in Setif, Algeria in February2024 and identified by Assist. Prof. Wafa Nouioua (Department of Botany, University of Setif 1, Algeria). Authenticated voucher specimen (No. S.c.2024-7) was deposited in the Herbarium of Botany, Department of Botany, University of Setif 1. Leaves were ground into powder and stored until use.

2.2. Preparation of extracts

Aqueous extract of *M. oleifera* leaves was obtained by decocting 20 g of finely powdered leaves in 200 ml of distilled water for 20 minutes. After filtration through Whatman paper, the obtained extract was dried and then stored in a small vial and kept in darkness until use. Methanol extract of *M. oleifera* leaves was prepared by macerating 20 g of finely powdered leaves in 80 ml of methanol for 24 hours in darkness. The macerate was then filtered through Whatman paper. This process was repeated on the residue using 50 ml of methanol. Subsequently, the dried extract was stored in darkness until use. (Boudoukha *et al.,* 2018).

2.3. Phytochemical screening

Phytochemical screening is based on the coloration and/or precipitation reactions of the chemical compounds present in the plant extract with chemical reagents (Ghedadba *et al.,* 2015). Phytochemical screening of both extracts of *M. oleifera* was carried out using standard methods (table 1) described by Yadav *et al*. (2014).

Table 1: Phytochemical tests for *M. oleifera* extracts

Phytoconstituents	Test	Observation
Polyphenols	1ml extract + $FeCl_3$ (2%)	Bleu blackish color/Dark green
Flavonoids	1ml extract + 0.5ml NH_4OH +0.5mlsulfuric acid	Yellow coloration
Tannins	1ml extract + $FeCl_3$	Green precipitate
Saponins	1ml extract + 2 ml H_2O	Froth appears
Quinones	1ml extract +0.5 ml sulfuric acid	Red coloration
Anthraquinones	1ml extract + HCl (2%)	Red precipitate
Terpenoids	1ml extract + 4 ml chloroform +sulfuric acid	Deep red coloration

2.4. Determination of Total Phenolic and Flavonoid Contents

Folin-Ciocalteu method was used to determine the amount of total phenolic compound (Singleton *et al.,* 1965). In test tubes, 200 µl of each diluted extract (aqueous or methanol extract) with 1 mg/ml concentration were mixed with 1 ml of diluted Folin-Ciocalteu reagent. After four minutes of incubation, 200 µl of sodium carbonate (20%) was added and incubated again for two hours in the darkness at room temperature. The total phenolic content was expressed as microgram gallic acid equivalent per milligram of extract (µg GAE/mg E). The absorbance of the reaction mixture was measured at 765 nm.

Total flavonoid content was determined using aluminum trichloride colorimetric method. One ml of each diluted extract was mixed with 1 ml of aluminum trichloride (20%). The mixture was then incubated for 30 minutes in darkness at room temperature. The absorbance of the reaction mixture was determined at 430 nm. The total flavonoid content was expressed as microgram quercetin equivalent per milligram of extract (µg QE/mg E). (Charoensin, 2014).

2.5. Analysis of extracts by HPLC-PDA

Individual phenolic compound contents of each extract used in the study were screened for 15 standard phenolic compounds.: Vanillic acid, Caffeic acid, Epicatechin, p-coumaric acid, Salicylic acid, Cinnamic acid, Rosmarinic acid, Quercetin, Chlorogenic acid, Apigenin-7-O-glucoside, Rutin, Naringenin, 4-hydroxybenzoic acid, Gallic acid and Ferulic acid.

Determination of quantitative phenolic compounds was carried out with HPLC-PDA detector (Shimadzu Nexera-i LC-2040C 3D). Phenylhexyl reverse phase column was used (3 µm, 4.6 x 150 mm; GL Sciences Inter Sustain). Pump program was given at Table 2. As the mobile phase, Solvent A is 0.1% formic acid in water and Solvent B is acetonitrile (Merck, HPLC grade). During the analysis, the mobile phase flow rate was set at 1 ml/min. The samples and standards injection volume are adjusted as 10 µl. The column temperature is set to 30 °C. For standards stock solutions and extract were prepared at 1000 mg/l concentration (Ataseven *et al.*, 2021). Results are calculated and given as mg/g extract.

Table 2 : HPLC gradient pump program.

Steps	**Flow rate (ml/min)**	**Time (min)**	**% Mobile Phase B (Acetonitrile)**	**% Mobile Phase A (%0.1 formic acid/Water)**
Step 1	1.00	0.01	5	95
Step 2	1.00	7	9.5	90.5
Step 3	1.00	20	17	83
Step 4	1.00	35	40	60
Step 5	1.00	40	0	100

2.6. Evaluation of the antioxidant effect

2.6.1. DPPH free radical scavenging assay

To evaluate antioxidant activity, DPPH free radical scavenging assay was used as described by (Lee *et al.,* 2002). One milliliter of each *M. oleifera* extract at various concentrations was combined with 1 ml of DPPH in methanol, and the mixture was shaken vigorously. After 30 minutes of incubation in darkness at room temperature, the amount of DPPH remaining was determined at 517 nm. The radical scavenging activity was calculated using the formula.

Radical scavenging activity (%) = ((DO control –DO sample) / DO control) x 100.

The control consisted of 1 ml of methanol and 1 ml of DPPH solution. The antioxidant activity of the plant extracts was expressed as IC_{50}, representing the concentration (in µg/ml) of extract required to inhibit the formation of DPPH radicals by 50%.

2.6.2. Total antioxidant capacity

To understand the total antioxidant capacity (TAC) of *M.oleifera,* a crucial procedure involves preparing a phosphomolybdate solution, which plays a vital role in assessing the

antioxidant potential of various samples. This method based on the reduction of Mo(IV) to Mo(V) followed by the appearance of a green colored phosphomolybdenum complex which shows maximum absorbance at 695nm (Umamaheswari and Chatterjee, 2008).

Initially, in test tubes 300 µl of each diluted extract were mixed with 3 ml of phosphomolybdate reactive, which is prepared by bleeding a specific quantity of sulfuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM). Tubes were screwed and incubated at 95°C in a water bath for 90 min. After cooling, the absorbance was recorded at 695 nm against the blank (preparing by mixing 300 µl of methanol with 3 ml of phosphomolybdate reactive). The total antioxidant capacity was expressed in equivalents of quercetin and ascorbic acid used as antioxidant standards (preparing by mixing 300 µl of each of them with 3 ml of phosphomolybdate reactive), representing 100% of the total antioxidant capacity. Values of the total antioxidant capacities of *M. oleifera* extracts were determined by the following equation:

$$\% \text{ inhibition} = (At / Ac) \times 100$$

At = absorbance of the sample at 695 nm

Ac = absorbance of quercetin or ascorbic acid.

2.5.3 Ferric reducing antioxidant power

The reducing power (FRAP) of both *M. oleifera* extracts was measured by the method of Oyaizu (1986) with a slight modification. According to this method, the reduction of $Fe^{3}+$ to $Fe^{2}+$ was determined by measuring the absorbance of the Perl`s Prussian Blue Complex. For this purpose, in test tubes 500 µl of each *M. oleifera* extract at various concentrations was mixed with 1.25 ml of phosphate buffer (0.1 M, pH 6.6) and 1.25ml (1%) of potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After incubation, the reaction was acidified with 1.25 ml of trichloro acetic acid (TCA 10%). Finally, 1.25 ml from each tube were mixed with equivolume of distilled water and 0.25 ml of ethanolic solution of $FeCl_3$ (0.1%).The absorbance was recorded at 700 nm. Quercetin and BHA were used as standard prepared by the same steps.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SD) of three independent experiments. The analysis was performed using a one-way analysis of variance followed by Tukey’s multiple comparison tests. The results were considered statistically significant when $^{*}P = 0.05$, $^{**}P = 0.01$, $^{***}P = 0.001$.

3. Results and discussion

3.1. Phytochemical screening

secondary metabolites, such as polyphenols, flavonoids, tannins and quinones, play a crucial role in the biological activities of medicinal plants, including hypoglycemic, antidiabetic, antioxidant, antimicrobial, anti-inflammatory and anticancer (Yadav *et al*., 2014). Both extracts of *M. oleifera* leaves contain polyphenols, tannins, flavonoids and quinones. While, terpenoids, saponins and anthraquinones were not detected by the phytochemical screening. Effectively, the results obtained by (Koruthu *et al.*, 2011) indicate the presence of polyphenols, flavonoids, and tannins in the methanol and aqueous extracts of the same plant. However, saponins were absent in the methanol extract.

3.2. Total Phenolic and Flavonoid Contents

Regarding the secondary metabolites in plants, polyphenol is one of them, known for their antioxidant activity. The total phenolic content of *M. oleifera* was determined using the Folin-Ciocalteu method for both aqueous and methanol extracts. The Folin-Ciocalteu reagent, a mixture of the phosphomolybdic and phosphotungstic acids, reacts with the polyphenol compounds in the sample. This reaction reduces acids, forming a blue molybdenum-tungsten complex (Fachriyaha *et al*., 2020).

Results showed that the methanol extract contained a higher amount of polyphenols (100.1052±10.24 µg GAE/mg E) compared to the aqueous extract (30.47±7.74 µg GAE/mg E).The results obtained by (Fachriyaha *et al*., 2020) and (Abdulkadir *et al.*, 2015) indicated a lower quantity of polyphenols in the methanol extract with values of 62.56±0.72 µg GAE/mg E and 32.83±1.19 µg GAE/mg E respectively, compared to our results. In contrast, (Belhi *et al*., 2018) reported higher values of polyphenols with 236.66±1.53 µg GAE/mg E for the methanol extract and 130.83±3.69 µg GAE/mg E for the aqueous extract. This difference was probably due to the differences in the varieties and growing sites of *M. oleifera* (Şanli and Karadoğan, 2017).

Flavonoid content of *M. oleifera* extracts was determined using the aluminum trichloride colorimetric method. The principle of this method is forming a stable acid complex with ortho-dihydroxy group on the A or B rings of flavonoids.(Fachriyaha *et al*., 2020) which resulting a color change. Results indicated that the amount of flavonoids in the aqueous extract was 15.16±2.16 µg QE/mg E, less than in the methanol extract, which was estimated at

52.85±0.46 µg QE/mg E (figure 1).Indeed, flavonoids values obtained in our results in the methanol extract were close to those indicated by (Vyas *et al*., 2015)and (Charoensin, 2014). However, the study conducted by (Belhi et al., 2018) showed higher values of flavonoid content in both extracts compared to our study. The differences in total phenolic content and total flavonoid content may be attributed to factors such as geographical regions with varying elevations, samples, treatment methods, the season of harvesting and leaf maturity (Dessalegn and Rupasinghe, 2021).

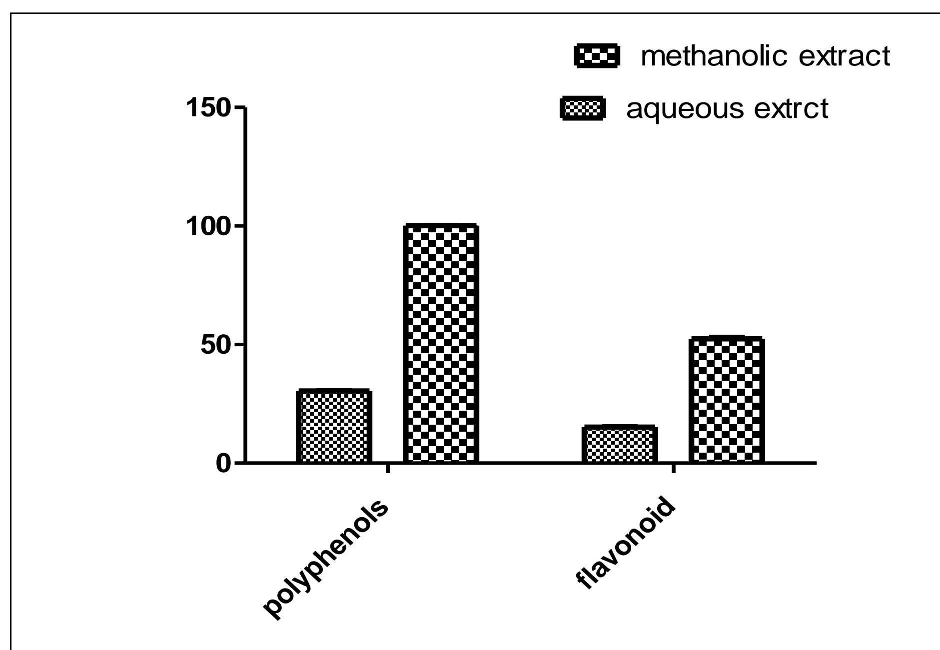

Figure 1: Histogram of total polyphenol and flavonoid in *M. oleifera* leaf extracts.

3.2.5. Analysis of extracts by HPLC-PDA

In HPLC investigation including 15 standard chemicals, we successfully identified chlorogenic acid in the *M. oleifera* aqueous extract at a concentration of 3. 085 mg/g extract and a retention time of 11.68 min. furthermore, in the methanol extract, rutin was identified at a concentration of 2.469 mg/g extract and a retention time of 23.86 min (figure 2, 3).

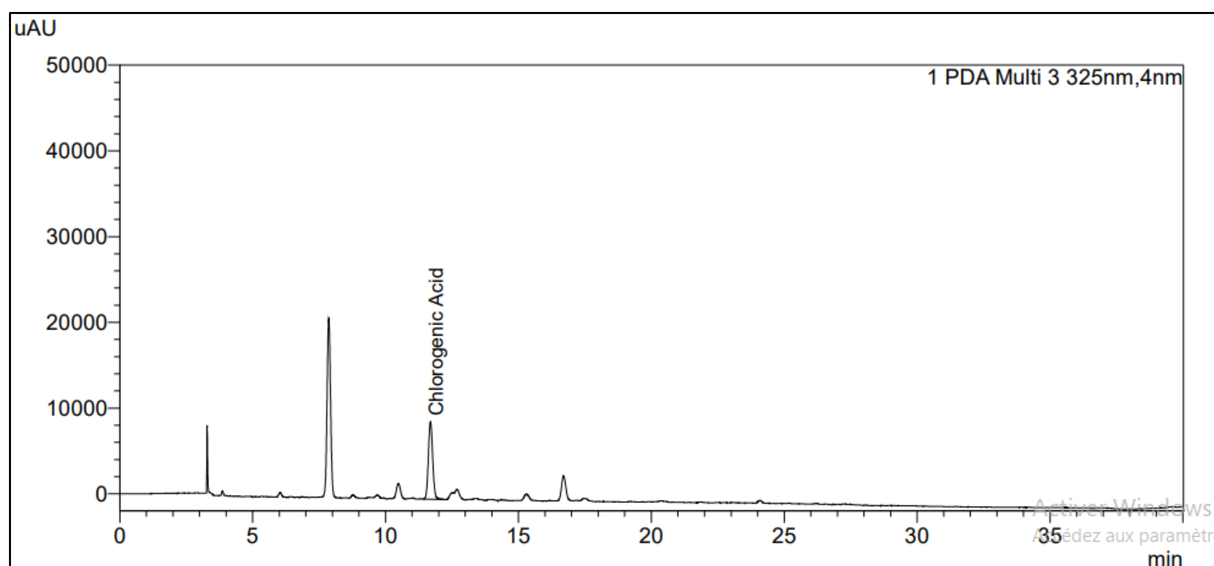

Figure 2 : *Moringa oleifera* aqueous exctract HPLC-PDA analysis chromatogram.

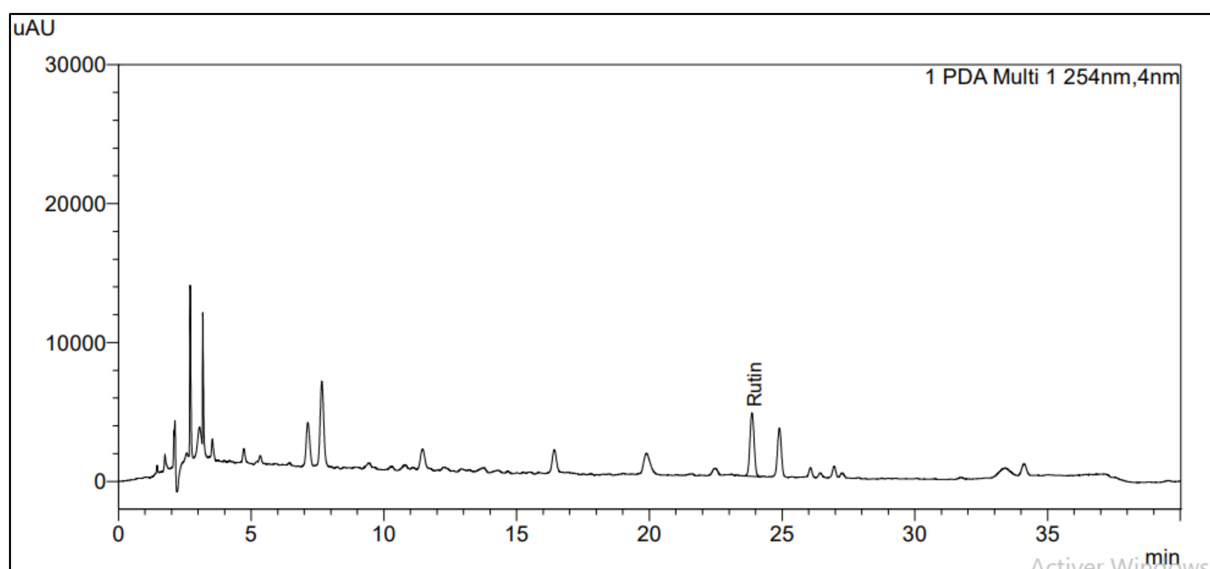

Figure 3: *Moringa oleifera* methanol exctract HPLC-PDA analysis chromatogram.

3.3. Evaluation of the antioxidant effect

3.3.1. DPPH radical scavenging assay

Antioxidants play an important role in food preservation by inhibiting oxidation processes and contributing to health promotion rendered by many dietary supplements, nutraceuticals and functional food ingredients.(Shahidi and Zhong, 2015)

The antioxidant activity of *M. oleifera* extracts was evaluated using methods including DPPH radical which has a hydrogen-free radical and shows a characteristic absorption at 517nm (Shih *et al*., 2011). The interaction between antioxidant compounds and DPPH radical is based

on the transfer of an electron or hydrogen atom to the DPPH radical, converting it into 1-1, diphenyl-2-picrylhydrazyl.The reduction of DPPH radicals causes a discoloration from purple color to pale yellow, indicating scavenging activity (Fitriana *et al.*, 2016).The scavenging effect of both extracts on DPPH radical was analyzed and compared to BHA, BHT, and Trolox as a positive control. According to the obtained results (figure 4,5), the methanol extract exhibited a significant (*P*<0.001) scavenging activity, with an IC_{50} of 70.24 ug/ml compared to aqueous extract with IC_{50} of 103.71ug/ml. Antioxidant standers were used in this study. Results showed that BHA, BHT and trolox exhibited a higher antioxidant activity with IC_{50} values of 12.53 ug/ml, 38.09 ug/ml 17.60 ug/ml, respectively. Vongsak *et al.* (2013) studied the antioxidant activity of *M. oleifera* leaves and found an IC_{50} 62.94 ug/ml for the methanol extract and IC_{50} of 123.44ug/ml for the aqueous extract, which are close to our results. Additionally, according to Nitesh *et al.* (2021), the free radical scavenging efficacy of *Moringa oleifera* leaf extracts was evaluated at varying concentrations. The aqueous extract exhibited an IC_{50} of 4.65 µg/ml, whereas the methanol extract showed a significantly lower IC_{50} of 1.83 µl/ml. This indicates a higher antioxidant potency for the methanol extract compared to the aqueous extract (Bhalla *et al.*, 2021). Several factors may contribute to this differences including solvent polarity, extraction efficiency and extraction conditions. The observed antiradical activity of *M.oleifera* may be due to polyphenol compounds present in the extract, especially flavonoids.

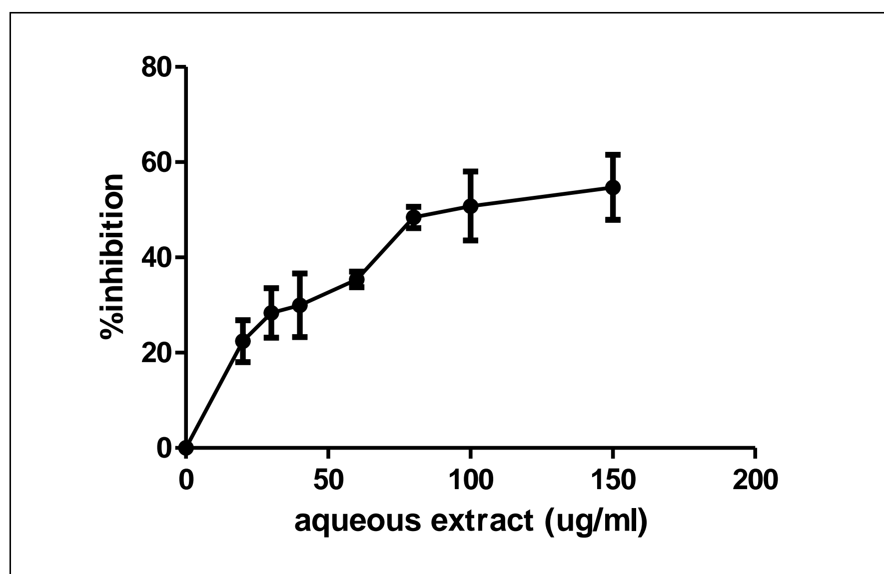

Figure 4: Percentage of DPPH inhibition as a function of the concentration of the aqueous extract of *M. oleifera* leaves

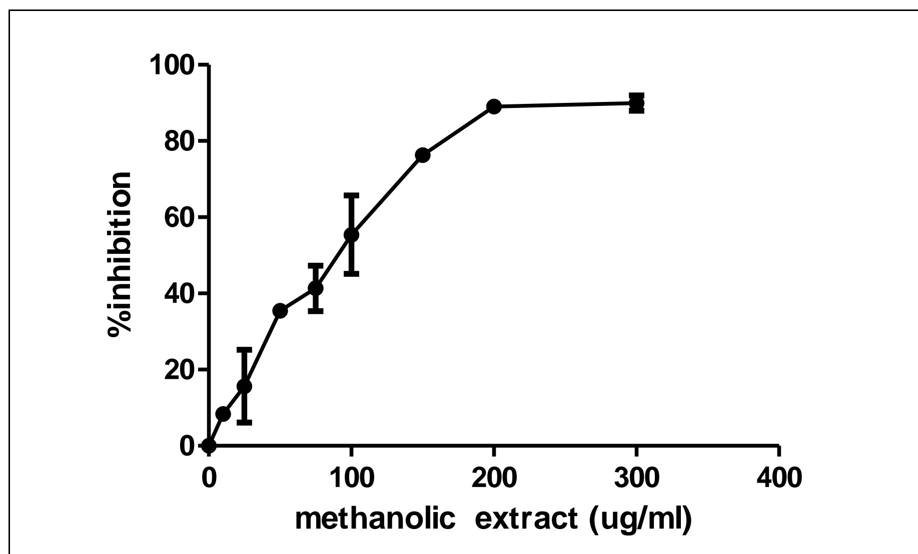

Figure 5:Percentage of DPPH inhibition as a function of the concentration of the methanol extract of *M. oleifera* leaves

3.4.2. Total antioxidant capacity

The results of the total antioxidant capacity (TAC) of *M. oleifera* using the phosphomolybdate reagent showed that the aqueous and methanol extracts exhibited significantly ($P< 0.001$) a lower percentage of reducing activity, 33.61± 2.895 % and 50.42± 4.236 % respectively, compared to ascorbic acid, which is considered to have 100% reducing activity (figure 6).

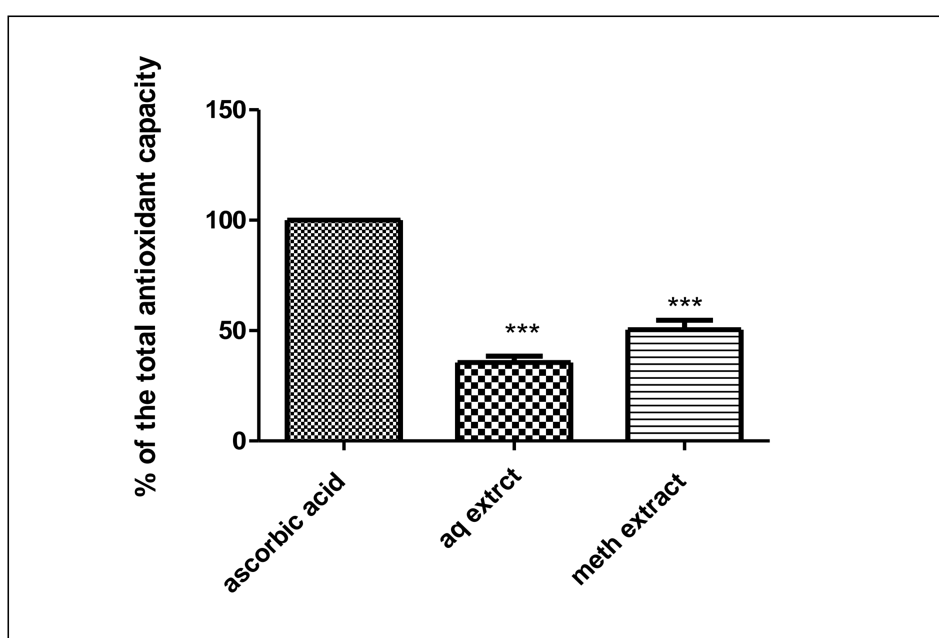

Figure 6: Histogram represents the total antioxidant capacity of the methanol and aqueous extract of *M. oleifera* compared to Ascorbic acid

When compared to quercetin, which also represents 100% reducing activity, both extracts of *M. oleifera* present a low percentage of antioxidant capacity (*P*<0.001), with 29.08±0.74% for the aqueous extracts and 43.62±3.65% for the methanol extract (figure 7). However, the methanol extract exhibited significant (*P* < 0.01) percentage inhibition higher than the aqueous extract. Thus, the antioxidant power depends on the type of solvent (Belhi *et al*., 2018).

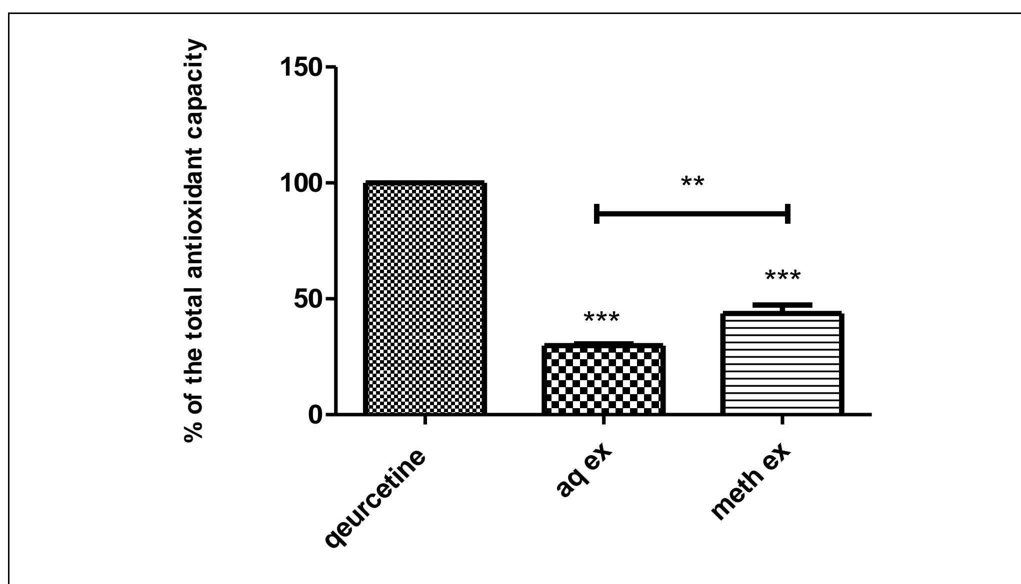

Figure 7: Histogram represents the total antioxidant capacity of the methanol and aqueous extract of *M. oleifera* compared to quercetin

3.4.3. Ferric reducing antioxidant power

The ferric reducing power (FRAP) method was used to evaluate the reducing activity of plant extracts. This antioxidant activity method is rapidly reproducible and easy to perform (Benzie and Strain, 1996).

Obtained results presented in Table 3, showed that the ferric reducing antioxidant power of both extracts of *M. oleifera* leaves exhibited a significant reducing power with EC_{50} of 0.773 mg/ml for the aqueous extract and 0.815 mg/ml for the methanol extract (figure 8). In fact, (Anigboro *et al*., 2019) reported that the aqueous extract of *M. oleifera* leaves from Nigeria have a lower potential of reducing power with EC_{50} 3.19 mg/ml. This difference may be due to the method of extraction or the geographical origin of plant (climate and soil) duration and storage conditions (Chong *et al*., 2020). BHA and quercetin (used as reference antioxidants), have a higher reducing power, compared to both extracts of *M. oleifera* with EC_{50} 0.209 mg/ml and 0.056 mg/ml, respectively (*P*<0.001).

Table 3: The values of EC_{50} and the antiradical activity of the aqueous and methanol extracts of *M. oleifera* compared with BHA and quercetin.

Sample	BHA	Quercetin	aqueous extract	Meth extract
EC_{50}(mg/ml)	0.209	0.056	0.773	0.815

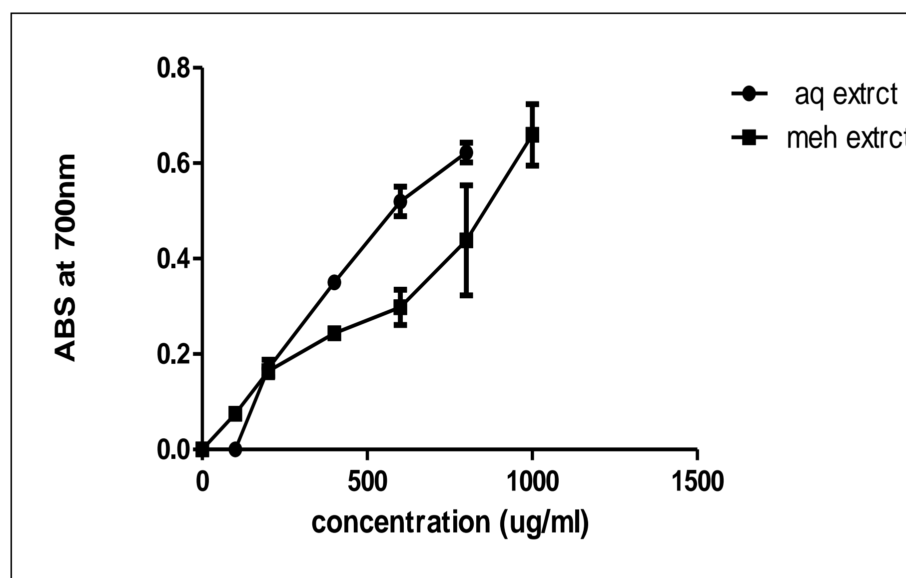

Figure 8: Calibration curve of the reducing power of aqueous and methanol extract of *M. oleifera* leaves

4. Conclusion

The present study demonstrated that both methanol and aqueous extracts contain polyphenols, tannins, flavonoids and quinones. The antioxidant activity of *M. oleifera* leaves were determined using DPPH, measurement of total antioxidant capacity (TAC) and FRAP method. Both extracts of *Moringa oleifera* showed a significant antioxidant activity. These findings highlight the potential of *M. oleifera* leaves as a source of valuable phytochemicals and justified its use in traditional medicine. Thus, some perspectives should be recommended such as optimization of extraction methods, isolation and characterization of new individual bioactive compounds from *M. oleifera* extracts and evaluation of *in vivo* and clinical studies to validate the therapeutic potential of *M. oleifera* extracts.

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