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Evaluation of the Antioxidant and Anti-Inflammatory Activities of *Moringa Oleifera* Lam Extracts and Determination of Their Active Components

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Abstract

Background: Natural products are attracting the attention of researchers due to their widespread use in pharmaceutical industries. Moringa oleifera is one of these plants with bioactivity and is used as an antioxidant. Objective: The current research was conducted to assess the anti-inflammatory and antioxidant efficacies of various Moringa leaf extracts and determine their active components. Materials and Methods: Different solvents (cold water, hot water, 70% ethanol, cold ethanol, and hot ethanol) were utilized to extract M. oleifera. The extracts were then analyzed with FTIR to determine the percentage of active compounds in each extract. Stabilization of the red blood cell membrane, denaturation protein inhibition, and antioxidant potential of the extracts were determined. Results: The FTIR analysis revealed the active components in the various extracts to be phenol compounds, as evidenced by a specific peak in certain regions. The hot ethanolic extract was found to have the highest amount of bioactive compound (89.33%) compared to other extracts. Also, the same extract had the highest activity in stabilizing red blood cells, protein inhibition, as well as radical scavenging. Conclusion: The findings of this investigation indicate that the hot ethanolic extract of M. oleifera is more effective in all the test activities compared to the other extracts.

Keywords: Antioxidant, *Moringa oleifera*, TPC, Protein denaturation, FTIR, RBCs membrane stabilization

1. Introduction

The Moringa plant (Moringa oleifera) is part of the Moringaceae family and is known by many names, including milk, oil milk, and horseradish [1]. Every plant part, including the leaves, bark, fruits, and roots, as well as the sap and flowers, can be used [2]. M. oleifera is available in the form of tablets, with a light flavor like that of tea. It can be mixed into smoothies and juices without changing the flavor. Also, moringa can be utilized to make tea. Moringa supplements are in the form of a light green powder that contains both the leaves and its seed pods [3]. Research published in 2018 [4] discovered that an extract from moringa reduced the risk and harm of arthritis and that the ethanolic extract of its leaves, depending on the dose, can relieve pain in mice with arthritis [as reported by 4].. Furthermore, moringa leaf extract can help with redness, rheumatoid arthritis pain, and fluid retention.

This research was conducted to assess the anti-inflammatory and antioxidant efficacies of various moringa leaf extracts using different solvents (water and/or ethanol), also to determine their active components.

Materials and Methods

Plant material

The plant materials were collected from a local

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market in Baghdad, Iraq.

M. oleifera Extracts

Different solvents, including cold and hot water, cold and hot ethanol, were employed to prepare moringa leaf extracts. The cold aqueous leaves extract was prepared according to Anesini and Perez [5]. One hundred grams of moringa powder was mixed with 500 ml of sterile distilled water. For 24 hours, the blend was incubated at 37°C. Then, It was centrifuged for 15 minutes (at 2,500 rpm), and kept at 4 °C in the refrigerator until use. The hot aqueous extract was made following the method described by Anesini and Perez [5]. Powder of moringa leaves (100 g) was added to 500 ml of sterile distilled water in a Soxhlet extraction apparatus for 24 hours. The filtrate was then collected in dark containers and stored at 4 °C. The technique [6] was utilized to prepare the cold ethanol extract [5]. One hundred (100 g) of moringa leaf powder and 600 ml of ethanol were thoroughly mixed. The mixture was incubated for 24 hours at 37 °C in a vibrating incubator. Then, it was centrifuged at 2,500 rpm for 15 minutes. The supernatant was obtained and kept at 4 °C in the refrigerator until needed. In preparing the hot ethanolic extract, the protocol of Ladd et al. was used [6]. Leaf powder from moringa (100 g) was added to ethanol (700 ml of C_2H_5OH) in a Soxhlet extraction apparatus and agitated for 24 hours. The filtrate was discarded and the resulting solution was kept at 4 °C until used. In preparing the 70% ethanolic extract, the protocol developed by Vongsak et al. was used [7]. One hundred grams of moringa leaves were weighed and placed in 70% ethanol, then incubated at 37°C for 24 hours in a vibrating incubator, after that the mixture was centrifuged for 15 minutes at 2,500 rpm, then the product was kept at 4°C until required.

Estimation of TPC (Total Phenolic Content)

Determination of TPC was carried out by a certain technique (Folin-Ciocalteau), as proceeded by Liu et al. [8], using Gallic Acid ($C_6H_2(OH)_3CO_2H$). An aliquot of 0.2 mL of the test extract sample was added to 0.8 mL sodium carbonate (20% conc.), after that add 1 mL of Folin-Ciocalteau, then allow the sample to stand for one hour, the absorbance was taken at 765 nm. The TPC was estimated as follows:
TPC (mg/l EAG) = Absorbance (Abs.)/Slope (m) + intercept constant (C)

The M.oleifera extracts that have been concentrated were then characterized by FTIR and assayed by the Folin-Ciocalteau's method.

FTIR

Infra red spectra were taken to all the extract solution to determine the active compounds; this was carried using Shimadzu IRPrestige-21 FTIR Spectroscopy

Assessment of Red Blood Cell (the stabilization of the membrane)

The suspension of RBCs was arranged by following the method described by Chanda and Juvekar [9]. Fresh human blood was mixed with Alsever's solution (1:1) and for 10 minutes was centrifuged at 3,000 rpm, the supernatant was then removed, and the sediments (RBCs) were rinsed three consecutive times with regular saline. The RBCs were diluted to 10% v/v in phosphate buffer. The reaction mixture contained 1 MI of extract at various concentrations (20–100 µg/Ml), aspirin (100 g/Ml), and 1 Ml RBC suspension (10% v/v). The reaction mixtures were incubated in a water bath at 56°C for 30 minutes, then, at room temperature and for 5 minutes the mixture was centrifuged at a certain rpm (2500 rpm). The absorbance was carried out at 560 nm. The following equation was used to calculate the percentage protection from heat-induced hemolysis [9].

Protection% = [(Ac – As)/Ac] × 100

Where Ac is the control absorbance, as is the aspirin/extract absorbance

Inhibition of Protein Denaturation Assay

The assay was carried out as depicted in 1968 by Mizushima and Kobayashi [11], with slight modifications, 1% of bovine serum albumin solution (500 mL) was added sequentially to 100 mL of extract at a range of concentrations (100–400 g/mL) as well as 400 g/mL of ibuprofen, then incubation of the resulting mixtures for 20 minutes at 37 °C, then heating for 20 minutes at 51 °C. At 660 nm, the turbidity was determined. The inhibition percent of protein denaturation was estimated according to the below formula:

Inhibition% = [(Ac – As)/Ac] × 100

Where Ac refers control absorbance, while As is referred to extract/ibuprofen absorbance.

Estimation of FRSP (Free Radical Scavenging Potential) via DPPH Assay

Following the procedure reported by Ruch et al [12], 10 mg/Ml of the moringa extract was made and diluted to reach concentrations ranged from 100 to 400 g/Ml. The standard used was ascorbic acid. The DPPH reagent was utilized to estimate the antioxidant potential of the extract. 1 Ml of DPPH in 0.3 Mm of methanol was mixed with 2.5 Ml of the moringa in different concentrations and kept at room temperature for 30 minutes in the dark. At 517 nm, all the samples were tested their absorbance, and methanol was used as a blank solution. The activity of DPPH inhibition was calculated from the formula below:

$$FRSA\% = [(Ac - As)/Ac] \times 100$$

Where Ac refers control absorbance, while As is the absorbance of the abstract

The remaining procedures were conducted at the Industrial Research and Development Authority, Veterinary Medicine Center, Iraq.

Statistical Analysis

The data obtained followed a normal distribution and mean ± standard deviation was presented as (SD). Among the groups, one-way ANOVA was used for comparing the parameters. This was followed by a post hoc test and the Least Significant Difference (LSD). The difference between groups was considered to be significant if $p < 0.05$.

Results and Discussion

The TPC results of the different extracts of Moringa oleifera are presented in Figure 1. The hot ethanolic extract had the highest concentration of TPC (46 mg/L) when compared to the other extracts, such as the cold ethanol, 70% ethanol, and cold aqueous. This observation is in support of the finding by [14], which discovered that a hot alcoholic extract of M. oleifera seed provided the highest TPC concentration.

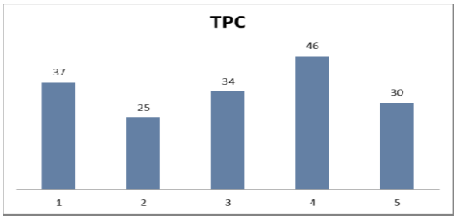

Figure 1: Total phenolic content of the different extracts of Moringa oleifera, (Extracts were prepared with different solvents, which include 1: Cold ethanol; 2: Cold water; 3: 70% ethanol; 4: Hot ethanol; 5: Hot water)

FTIR Analysis

The FTIR spectrum of M. oleifera leaf extracts revealed that it contains a variety of natural compounds (Figure 2). The different leaf extracts of M. oleifera with different solvents such as cold ethanol, cold water, 70% ethanol, cold and hot water, resulted in different natural compounds. Information on the functional groups of the different extracts was obtained from the FTIR analysis. It was observed that the FTIR spectrum of the leaf extracts revealed a broad signal at ~33170.67 cm^{-1}. The FTIR spectrum of the different M. oleifera extracts showed sharp signals at 2159.81, 1604.77, 1511.24, 1450.34, 1303.72, 1047.66, and 920.31 cm^{-1}. Analysis of phytochemicals of the extracts revealed that all the extracts contained O-H and C=O functional groups, which could be derived from polar natural compounds, as evidenced by a strong signal. On the other hand, the aliphatic and aromatic carbons were detected as distinct signals at 1450-1500.11 and 1159.81 cm^{-1}, respectively. Alkaloids, phenolics, carbohydrates, saponins, polyphenols, proteins, flavonoids, and amino acids, were found in the phytochemical analysis, but in varying degrees of transmittance (%) [15 and 16], the extracts of moringa leaves and the solvents were compared (Figure 1), which shows that higher TPC is observed in the hot alcoholic. Five FTIR spectrums were carried out, each extract has its own spectra, figure 2 (A, B, C, D and E), and tables 1, 2, 3, 4, and 5 as below, while the functional groups were analyzed according to [16, 17, 18].

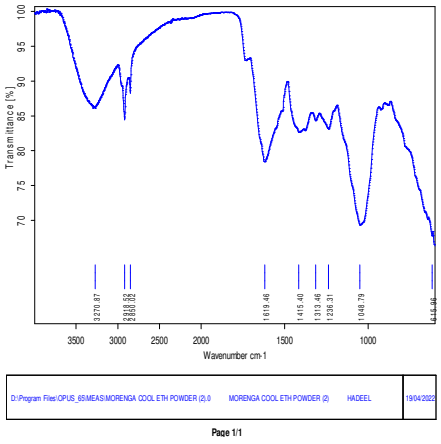

Figure 2 A: Cold ethanol

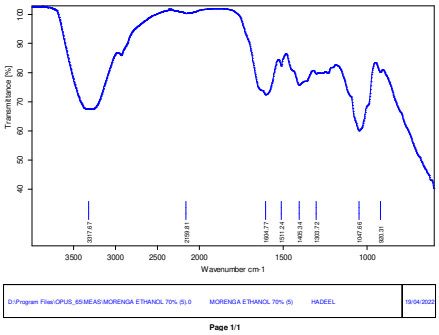

Figure 2 B: Cold water

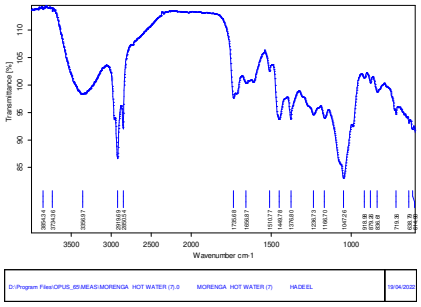

Figure 2 C: 70% ethanol

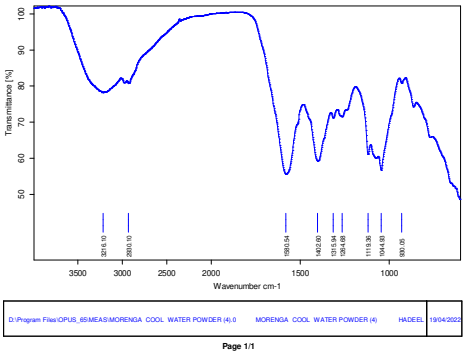

Figure 2D: Hot ethanol

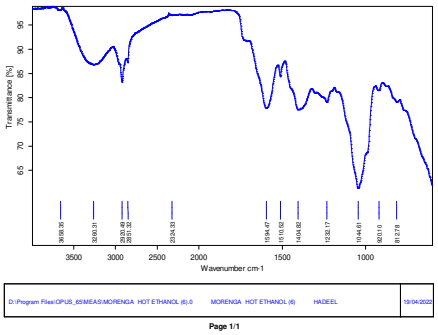

Figure 2 E: Hot water

Table 1

Functional group and their quantitative frequencies for cold alcoholic extract of Moringa leaves. Data was adopted from reference figure 2 A [17]

No.	Frequency ranges (cm^{-1})	Frequency peak values (cm^{-1})	Vibration/bond	Specific functional group	Chemical compound
1	3400–3250	3270.87	N–H stretch	1°, 2° amines and amides	Amines and amides
2	3000–2850	2918.52	C–H stretch	Alkanes	Aliphatic
3	3000–2850	2850.02	C–H stretch	Alkanes	Aliphatic
4	1680–1620	1619.46	C=Cstretch	Alkene	Aliphatic
5	1420–1410	1415.40	C-H Vinyl	Alkene	Aliphatic
6	1360–1310	1313.46	C-N stretch	Aromatic amino	Aromatic tertiary amine
7	1300–700	1236.31	C-C	Saturated Aliphatic (alkene/alkyl)	Methyne (>CH–)
8	1150–1000	1048.79	C-F stretch	Aliphatic organohalogen compound	Aliphatic fluoro compounds

Table 2

Functional group and their quantitative frequencies for cold water extract of Moringa leaves (figure 2 B) [17]

No.	Frequency ranges (cm^{-1})	Frequency peak values (cm^{-1})	Vibration/bond	Specific functional group	Chemical compound
1	3400–3200	3216.10	OH stretch	Alcohol and hydroxy	Normal ‘‘polymeric
2	3000–2850	2930.10	C–H stretch	Alkanes	Aliphatic
3	1650–1550	1580.54	N-H bend	Secondary amino	Secondary amine
4	1410–1310	1402.60	O-H bend	Alcohol and hydroxy compound	Phenol or tertiary alcohol,
5	1350–1260	1315.94	O-H bend	Alcohol and hydroxy compound	Primary or secondary
6	1270–1230	1264.68	aryl -O stretch	Ether and oxy compound	Aromatic ethers
7	1150–1000	1119.36	C-F stretch	Aliphatic organohalogen compound	Aliphatic fluoro compounds
8	1150–1000	1044.93	C-F stretch	Aliphatic organohalogen compound	Aliphatic fluoro compounds
9	1005–925	930.05	> CH_2	Methylene	Cyclohexane ring vibrations

Table 3
Functional group and their quantitative frequencies for 70% alcohol water extract of Moringa leaves Figure 2 C [17, 18]

No.	Frequency ranges (cm^{-1})	Frequency peak values (cm^{-1})	Vibration/bond	Specific functional group	Chemical compound
1	3320–3310	3317.67	C-H stretch	Alkyne	Acetylenic(alkyne)
2	2270–1940	2159.81	C≡C stretch	Alkynes	Aliphatic
3	1650–1590	1601.77	N-H bend	Primary amino	Primary amine
4	1555–1485	1511.24	(N–O asymmetric stretch)	Nitrogen-oxy compounds	Aromatic nitro compounds
5	1410–1310	1405.34	O-H bend	Alcohol and hydroxy compound	Phenol or tertiary alcohol
6	1310–1290	1303.72	C-H bend	alkene	Vinylidene
7	1150–1000	1047.66	C-F stretch	Aliphatic organohalogen compound	Aliphatic fluoro compounds
8	1100–900	920.31		Common inorganic ions	Silicate ion
No.	Frequency ranges (cm^{-1})	Frequency peak values (cm^{-1})	Vibration/bond	Specific functional group	Chemical compound
1	3320–3310	3317.67	C-H stretch	Alkyne	Acetylenic(alkyne)
2	2270–1940	2159.81	C≡C stretch	Alkynes	Aliphatic
3	1650–1590	1601.77	N-H bend	Primary amino	Primary amine
4	1555–1485	1511.24	(N–O asymmetric stretch)	Nitrogen-oxy compounds	Aromatic nitro compounds
5	1410–1310	1405.34	O-H bend	Alcohol and hydroxy compound	Phenol or tertiary alcohol
6	1310–1290	1303.72	C-H bend	alkene	Vinylidene
7	1150–1000	1047.66	C-F stretch	Aliphatic organohalogen compound	Aliphatic fluoro compounds
8	1100–900	920.31		Common inorganic ions	Silicate ion

Table 4
Functional group and their quantitative frequencies for hot alcoholic extract of Moringa leaves figure 2 D [17. 18]

No.	Frequency ranges (cm^{-1})	Frequency peak values (cm^{-1})	Vibration/bond	Specific functional group	Chemical compound
1	3645–3630	3658.35	O-H stretch	Alcohol and hydroxy compound	Primary alcohol
2	3570-3200	3260.31	H-bonded O-H stretch	Alcohol and hydroxy compound	Hydroxy group
3	2935–2915	2920.49	C-H Stretch	Methylene (>CH_2)	Methylene
4	2865–2845	2851.32	C-H Stretch	Methylene (>CH_2)	Methylene
5		2324.33		Unknown	Unknown
6	1615–1580	1594.47	C=C-C stretch	Aromatic ring (aryl)	Aromatic ring
7	1555–1485	1510.52	(N–O asymmetric stretch)	Nitrogen-oxy compounds	Aromatic nitro compounds
8	1410–1310	1404.82	O-H bend	Alcohol and hydroxy compound	Phenol or tertiary alcohol
9	1270–1230	1232.17	aryl -O stretch	Ether and oxy compound	Aromatic ethers
10	1150–1000	1044.61	C-F stretch	Aliphatic organohalogen compound	Aliphatic fluoro compounds
11	1100–900	920.10		Common inorganic ions	Silicate ion
12	860–800	812.78	C-H	Aromatic ring (aryl)	1,4-Disubstitution

Table 5
Functional group and their quantitative frequencies for hot water extract of Moringa leaves figure 2 E [18]

No.	Frequency ranges (cm^{-1})	Frequency peak values (cm^{-1})	Vibration/bond	Specific functional group	Chemical compound
1		3854.34		Unknown	Unknown
2		3734.36		Unknown	Unknown
3	3570–3200	3356.97	H-bonded OH stretch	Alcohol and hydroxy compound	Hydroxy group
4	2935–2915	2919.69	C-H Stretch	Methylene (>CH_2)	Methylene
5	2865–2845	2850.54	C-H Stretch	Methylene (>CH_2)	Methylene

6	1750–1725	1735.68		Carbonyl compound	Ester
7	1680–1620	1656.87	C=C stretch	Alkene	Alkenyl
8	1555–1485	1510.77	(N–O asymmetric stretch)	Nitrogen-oxy compounds	Aromatic nitro compounds
9	1490–1410	1449.78		Common inorganic ions	Carbonate ion
10	1420–1370	1376.80		Sulfur-oxy compounds	Organic sulfates
11	1270–1230	1236.73	aryl -O stretch	Aromatic ethers	Ether and oxy compound
12	1190–1130	1166.70	C-N stretch	Secondary amino	Secondary amine
13	1050–990	1047.26	(P-O-C stretch)	Phosphorus-oxy compounds	Aliphatic phosphates
14	1100–900	918.98		Common inorganic ions	Silicate ion
15	890–820	879.26	C-O-O- stretch	Ether and oxy compound	Peroxides
16	890–820	836.61	C-O-O- stretch	Ether and oxy compound	Peroxides
17	720–590	719.36	OH out-of-plane bend	Alcohol and hydroxy compound	Alcohol
18	680–610	638.79	C-H bend	Acetylenic(alkyne)	Alkyne
19	680–610	614.90	C-H bend	Acetylenic(alkyne)	Alkyne

The stabilization of the RBCs membrane

Table 6 depicts the effectiveness of moringa extracts in stabilizing the red blood cell membrane. It was discovered that the efficacy of red blood cells increased significantly with an increasing concentration of extract, with the highest concentration (100 µg/ml) having the highest efficacy of all extracts when compared to aspirin. Also, it was observed that hot ethanolic extract had the highest efficiency when compared to the other extracts. The level of efficacy is in the order: hot aqueous extract (65) < 70% ethanolic extract (65.33) < cold ethanolic extract (72) < cold aqueous extract (84.33) <hot ethanolic extract (89.33%). Furthermore, there are significant differences in the concentrations of all the extracts.

Table 6
The table showed heat-induced hemolysis for moringa leaves extract

Concentration (µg/ml)	Cold Alcoholic	Cold Water	70% Alcoholic	Hot Alcoholic	Hot Water	P value
20	28 .00± 1 A a	35 ± 2 A b	26.33 ± 2.52 A a	37 ± 1 A b	32 ± 2 A b	Sig
40	32.33 ± 2.08 A a	43 ± 3 B b	37.67 ± 0.58 B c	47 ± 2.65 B b	42.67 ± 2.08 B b	Sig
60	46.33 ± 1.53 B a	50 ± 2.65 C a	47 ± 2.65 C a	58 ± 2.65 C b	57.33 ± 2.08 C b	Sig
80	52.67 ± 1.53 C a	55.33 ± 2.51 D a	55.33 ± 1.53 D a	79.33 ± 2.08 D b	74 ± 4.58 D c	Sig
100	65.33 ± 5.03 D a	65 ± 2.64 E a	72 ± 1.53 E b	89.33 ± 2.08 E c	84.33 ± 2.08 E c	Sig
Aspirin 100	80 ± 0 E a	85 ± 0 Fa	85 ± 0 F a	85 ± 0 F a	85 ± 0 E a	not sig

Inhibition of Protein Denaturation

The results revealed significant differences between the extract types, and it was discovered that increasing the extract concentration increased the percentage of protein denaturation inhibition. The hot aqueous extract had the highest inhibition efficiency compared to the remaining extracts. The inhibition efficiency is in the order of hot aqueous extract (80.67) > hot ethanolic extract (76) > 70% ethanolic extract (66.67) > cold ethanolic extract (61.67) > cold aqueous extract. It was discovered that there was a noteworthy difference between the concentrations of all the extracts [19].

Free Radical Scavenging Potential via DPPH Assay

Figure 3 shows the outcomes of the FRSP of moringa extracts by the DPPH technique. The highest activity as an antioxidant was obtained in the 70% ethanolic extract compared to the other extracts and the standard ascorbic acid. The inhibition percentage is in the order of 70% ethanolic extract (84.33%) > hot aqueous extract (74.67%) > hot ethanolic extract (72.67%) > cold ethanolic extract (67.67%) > cold aqueous extract (59.67%). The DPPH is a free radical method for detecting the ability of a substance to act as a free radical scavenger [20]. Furthermore, the

technique also revealed that the 70% ethanolic extract may contain many compounds that can donate hydrogen to scavenging free radicals to remove the odd electrons, which are accountable for the radical's reactivity.

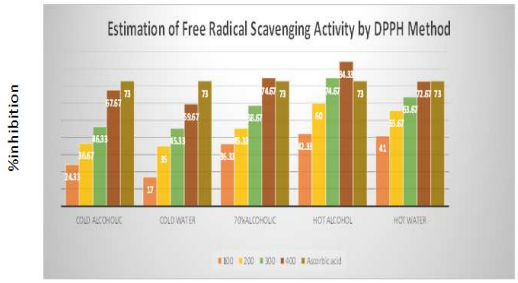

Figure 3: Estimation of FRSA activity of *Moringa oleifera* extracts by DPPH technique

Conclusion

The outcomes of this study revealed that moringa leaf extracts contain phenol, which caould be considered as an anti-microbial compound. The highest amount (46 mg/L) of TPC was found in the hot ethanolic extract. Furthermore, the hot ethanolic extract has the highest activity in red blood cell stabilization, and protein inhibition, and is a very active antioxidant in removing free radicals.

Acknowledgments

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