Anti-inflammatory and antioxidant activities of *Moringa peregrina* Seeds

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ABSTRACT

The present study was carried out to evaluate the anti-inflammatory and antioxidant activities of *Moringa peregrina* seeds ethanolic and aqueous extracts. The anti-inflammatory effect *Moringa peregrina* extracts was examined in rats, using fresh egg albumin-induced oedema, and diclofenac (100 mg/kg) was used as reference drug for comparison. The methods selected to design a convenient system to assess the antioxidant activity of *Moringa peregrina* extracts including, reducing power, chelating activity on Fe^{2+}, free radical-scavenging, total antioxidant, superoxide radical scavenging, hydrogen peroxide scavenging and hydroxyl radical scavenging activities in an attempt to understand its mechanism of action, which may pave the way for possible therapeutic applications. In addition, the results were compared with natural and synthetic antioxidants, such as α- tocopherol, ascorbic acid, butylated hydroxytoluene(BHT), butylated hydroxyanisole (BHA) and trolox. The results indicated that *Moringa peregrina* ethanolic and aqueous extracts (100-300 mg/kg p. o.) also dose-dependently and significantly inhibited (p < 0.05-0.001) fresh egg albumin-induced acute inflammation. Ethanolic and aqueous extracts of *Moringa peregrina* exhibited a strong reducing power, Fe^{2+} chelating effect, free radical scavenging activity, hydrogen peroxide scavenging ability, and hydroxyl radical scavenging activity. Antioxidant activity of the ethanolic and aqueous extracts increased with increased concentrations in the range of 20-140µg/ ml the extracts also exhibited a strong superoxide radical scavenging activity. This study showed that *Moringa peregrina* extracts exhibited antioxidant activity in all tests and the extracts could be considered as a source of natural antioxidants. Taken together, *Moringa peregrina* has potential as an anti-inflammatory and antioxidant agent against inflammation and free radicals and deserves clinical trial in the near future.

Key words: *Moringa peregrina*, anti-inflammatory, antioxidant activity, α- tocopherol, ascorbic acid and butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA).

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DOI: 10.5530/ax.2011.2.10

INTRODUCTION

The exogenous chemicals and endogenous metabolites in the body produce reactive oxygen species (ROS), which are capable of oxidizing various biomolecules resulting in tissue damage and cell death. Free radicals have been implicated in the cause of several diseases such as liver cirrhosis, atherosclerosis, cancer and diabetes and play an important role in ageing. Oxidative stress can also contribute to the development of neuro-degenerative disorders, such as Alzheimer's and Parkinson's as well as other diseases. These free radicals attack unsaturated fatty acids of biomembranes, resulting in lipid peroxidation and desaturation of proteins and DNA, causing a series of deteriorative changes in the biological systems leading to cell inactivation.[1] Many antioxidant enzymes and compounds present in the cell, mitigate the oxidative stress due to free radicals, by dismutating these free radicals species or by converting these radicals into a less effective forms.[2] Antioxidant supplements or antioxidant-rich food are used to help the human body reduce oxidative damage from free radicals and active oxygen species.[3] Other synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and trolox are widely used as antioxidants in the pharmaceutical and food industry. However, they have been shown to have toxic and/or mutagenic effects.[2] Because of their toxicity, the development and isolation of natural antioxidants

from plant species, such as polyphenols are in progress.[4] Many plant extracts are important constituents of human diet and have been suggested to have beneficial function in human health.[5-8] Phenolic compounds constitute one of the largest groups of phytochemicals which present in foods such as flavonoids and other phenolic compounds have been identified to be potent antioxidants as well as anticarcinogenic agents.[9,10] Polyphenols exist in many plants and are especially abundant in the *Moringa* tree,[11] whose dried leaves are used as antioxidant. *Moringa* and polyphenol-enriched plant extracts have no known toxicity and represent a promising antioxidant activity.[12] *Moringa oleifera* belongs to the *Moringae* family, commonly known as a drumstick tree that is native to tropical Africa and India and widely naturalised and cultivated in many countries including Malaysia.[13] A literature survey indicated that the presence of quercetin flavonoids,[14] sterols,[15] tocopherols (γ and α), β-carotene and vitamin C. In addition, Plant family is rich in essential sulfur amino acids; methionine and cysteine,[16] essential oils,[17] antioxidants,[18] glucosinolates and isothiocyanates.[19]

Besides being a source of nutrients,[20] studies have also demonstrated beneficial properties of this tree such as anticoagulant activity for water and waste water treatment,[21] anti-inflammatory and antinociceptive activities[22] as well as wound healing property.[23] In ayurvedic practice of India, *Moringa* has been used for treatment of various liver disorders.[24] We have recently reported that *Moringa oleifera* may have protected the liver in hepatotoxicity via increasing the level of glutathione.[25,26] As an extension of our interested research program in the extraction and therapeutic evaluation of rare medicinal plants,[5-8] we report herein, a facile route to explain the anti-inflammatory and antioxidant effects of *Moringa peregrina* extracts which may pave the way for possible therapeutic application.

MATERIALS AND METHODS

Chemicals

α- tocopherol, ascorbic acid, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and trolox were from Sigma, USA. All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant material

The seeds of *M. Peregrina* were purchased from the herbalist. The taxonomical features of the plant were kindly confirmed by *Dr.* A. A. Abd El Meged, *Prof.* of Plant Taxonomy, Agriculture research center, Cairo, Egypt. Voucher specimens were kept in the department of Pharmacognosy, Faculty of Pharmacy, October 6 University.

Preparations of the extracts

Preparation of ethanolic extract: *M. peregrina* seeds (500 g) were crushed to coarse powder and extracted exhaustively in a Soxhlet with 70% ethanol. The extract was concentrated under reduced pressure to yield viscous mass. The ethanolic extract was kept in airtight containers in a deep freeze maintained at 4 ºC until the time of further use.

Preparation of aqueous extract: The aqueous extract of *M. peregrine* seeds was prepared by dissolving a known amount of *M. peregrine* seeds powder in (warm distilled water 60-70 ºC) using a magnetic stirrer. It was then filtered and evaporated to dryness under reduced pressure. An aqueous suspension, which is the form customarily, used in folk medicine, was prepared to facilitate easy handling.

Phytochemical Screening: A phytochemical analysis of seeds of *M. peregrine* was conducted for the detection of alkaloids, cardiac glycosides, flavonoids, tannins, anthraquinones, saponins, volatile oil, coumarins and cyanophoric glycosides.[27]

METHODS

I. Evaluation of Anti-inflammatory Property

The rats used were divided into three broad (A, B, C and D) experimental groups of 8 rats per group. Group A rats were used as controls, and each animal in this group (A) received distilled water (3 ml/kg i.p.) only. Group B 'test' rats received *Moringa peregrine* ethanolic extract (100-300 mg/kg i.p.). Group C 'test' rats received *Moringa peregrina* aqueous extract (100-300 mg/kg i.p.). Group D 'test' rats received diclofenac (100 mg/kg i. p.). The rat hind paw oedema was used as a model of acute inflammation. Rat hind paw oedema was induced by intra-plantar injection of fresh egg albumin (0.5 ml/kg).[28] Acute inflammation of the right hind paw was induced in each of the rats by injecting 0.5 ml/kg of fresh egg albumin into the surface of the right hind paw. Pedal inflammation (oedema) was always evident within 5-8 min

following fresh egg albumin (0.5 ml/kg) injection. Linear diameter of the injected paw was measured (with a transparent millimeter ruler) for 3 h at 30-minute intervals after the administration of the fresh egg albumin. Increases in the linear diameter of the right hind paws were taken as indicators of paw oedema. Oedema was assessed in terms of the difference in the 'zero time' (C_o) linear diameter of the injected right hind paw, and its linear diameter at 'time t' [(C_t)-that is, 30, 60, 90, 120 and 180 min] following fresh egg albumin administration. The increases in the right hind paw diameters induced by injections of fresh egg albumin were compared with those of the contra-lateral, non-injected left hind paw diameters.[29] *Moringa peregrina* ethanolic and aqueous extracts were separately administered at doses of 100-300 mg/kg (i.p.) to each of the rats in the 'test' Groups B and/or C, 20 min before inducing inflammation with the injection of fresh egg albumin. Rats in the reference, comparative 'test' Group D received diclofenac (100 mg/kg i. p.); while rats in the 'control' Group A received distilled water (3 ml/kg i. p.) only.

Percentage inflammation (oedema) was calculated from the formula:

$$\frac{C_o}{C_t} \times 100$$

Percentage inhibition of the oedema was calculated from the formula:

$$\frac{C_o - C_t}{C_o} \times 100$$

[where C_o is the average inflammation (hind paw oedema) of the 'control' Group A rats at a given time; and C_t is the average inflammation of the (Groups B and/or C) plant's extracts or (Group D) diclofenac-treated rats at the same time].

II. Evaluation of Antioxidant Property

1. Determination of reducing power: The reducing power of ethanolic and aqueous extracts of *Moringa peregrina* was measured according to the method of Oyaizu.[30] Various concentrations of extracts (20-140 µg) in 1 ml of distilled water were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 7.6) and 2.5 ml potassium ferricyanide [K_3 $Fe(CN)_6$] (1%, w/v), and then the mixture was incubated at 50 ºC for 30 min. Afterwards, 2.5 ml of trichloroacetic acid (10%, w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of upper-layer solution was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$ (0.1%, w/v), and the absorbance was measured at 700 nm. α- tocopherol, BHA and BHT were used as standard antioxidants. Higher absorbance of the reaction mixture indicated greater reducing power.

2. Determination of chelating activity on Fe^{2+}: The chelating activity of ethanolic and aqueous extracts of *Moringa peregrina* on ferrous ions (Fe^{2+}) was measured according to the method of Decker and Welch.[31] Aliquots of 1 ml of different concentrations (0.25, 0.50, 0.75 and 1.0 mg/ml) of the extracts were mixed with 3.7 ml of deionized water. The mixture was incubated with $FeCl_2$ (2 mM, 0.1 ml) for 5, 10, 30 and 60 min. After incubation the reaction was initiated by addition of ferrozine (5 mM and 0.2 ml) for 10 min at room temperature, and then the absorbance was measured at 562 nm in a spectrophotometer. A lower absorbance indicates a higher chelating power. The chelating activity of extracts on Fe^{2+} was compared with that of EDTA at a level of 0.037 mg/ml. Chelating activity was calculated using the following formula:

Chelating activity (%) =

$$\left[1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100\right]$$

Control test was performed without addition of extracts.

3. Determination of free radical-scavenging activity: The free radical scavenging activity of ethanolic and aqueous extracts of *Moringa peregrina* was measured with 1,1-diphenyl-2-picrylhydrazil ($DPPH^{\cdot}$) using the slightly modified methods of Brand William et al.[32] and Takashira and Ohtake.[33] Briefly, $6x10^{-5}$ mol/l $DPPH^{\bullet}$ solution in ethanol was prepared and 3.9 ml of this solution was added to 0.1 ml of the extracts (2- 6 mg/ml) and trolox solution (0.02- 0.06 mg/ml). The mixture was shaken vigorously and the decrease in absorbance at 517 nm was measured at 5, 10, 30 and 60 min. Water (0.1 ml) in place of extracts was used as control. The percent inhibition activity was calculated using the following equation:

$$\text{Inhibition activity (\%)} = \left[\frac{(A_0 - A_1)}{A_0 \times 100}\right],$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of extracts.

4. Determination of total antioxidant activity determination: The antioxidant activity was determined according to the thiocyanate method with slight modifications.[34] For the stock solution, 10 mg of

ethanolic and aqueous extracts of *Moringa peregrina* was dissolved in 10 ml water. Then the solution of extracts or standards samples (tocopherol, trolox, BHA and BHT) [100mg/l] in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.6) was added to 2.5 ml of linoleic acid emulsion. Fifty ml linoleic acid emulsion contained Tween-20 (175 μg), linoleic acid (155 μl) and potassium phosphate buffer (0.04 M, pH 7.6). On the other hand, 5.0 ml of control contained 2.5 ml of linoleic acid emulsion and 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.6). Each solution was then incubated at 37 °C in a glass flask in the dark. At 24 h intervals during incubation, 0.1 ml of this incubation solution was added to 4.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after 0.1 ml of 0.02 M $FeCl_2$ in 3.5% (w/v) HCl was added to the reaction mixture, the absorbance of the red colour was measured at 500 nm in a spectrophotometer. The solutions without added extracts or standards were used as the control. The inhibition of lipid peroxidation in percentage was calculated by the following equation:

$$\text{Inhibition \%} = \left[\frac{(A_0 - A_1)}{A_0 \times 100}\right],$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of extracts or standards.

5. Determination of superoxide radical scavenging activity: Measurement of superoxide anion scavenging activity of ethanolic and aqueous extracts of *Moringa peregrina* was based on the method described by Liu et al.[35] Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide anion was generated in 3 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 μM) solution, 1 ml of NADH (78 μM) solution and different concentrations (0.1-1.25 mg/ml) of sample solution. The reaction was started by adding 1 ml of PMS-NADH solution (10 μM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and absorbance at 560 nm was recorded against blank samples in a spectrophotometer. Trolox, ascorbic acid and BHA were used as standard samples (0.1-1.25 mg/ml).

6. Determination of hydrogen peroxide scavenging activity: Ethanolic and aqueous extracts of *Moringa peregrina* (100 μg/ml) was dissolved in 3.4 ml of 0.1 M phosphate buffer (pH 7.6) and mixed with 0.6 ml of 43 mM hydrogen peroxide solution. The absorbance value (at 230 nm) of the reaction mixture was recorded after 40 min. For each concentration, a separate blank sample was used for background subtraction.[36] α- tocopherol, BHT and BHA (100 μg/ml) were used as standard antioxidants. The solutions without added extracts or standards were used as the control. The percentage of scavenged hydrogen peroxide of extracts and standard compounds was calculated using the following equation:

$$\text{Scavenged } H_2O_2\ \% = \left[\frac{(A_0 - A_1)}{A_0 \times 100}\right],$$

Where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of extracts and standards.

7. Determination of hydroxyl radical scavenging activity: The effect of hydroxyl radical was assayed by using the 2-deoxyribose oxidation method.[37] 2-Deoxyribose is oxidized by the hydroxyl radical that is formed by the Fenton reaction and degraded to malondialdehyde. The reaction mixture contained 0.45 ml of 0.2 M sodium phosphate (pH 7.6), 0.15 ml of 10 mM 2-deoxyribose, 0.15 ml of 10 mM $FeSO_4$-EDTA, 0.15 ml of 10 mM hydrogen peroxide, 0.525 ml of distilled water and 0.075 ml (20-120 μg/ml) of extracts solution in a tube. The reaction was started by the addition of hydrogen peroxide. After incubation at 37 °C for 4 h, the reaction was stopped by adding 0.75 ml of 2.8% (w/v) trichloroacetic acid and 0.75 ml of 1.0% (w/v) of thiobarbituric acid. The mixture was boiled for 10 min, cooled in ice and then measured at 520 nm. The reaction mixture not containing extracts was used as the control. Trolox, ascorbic acid, BHT and BHA (20-120 μg/ml) were used as standard antioxidants. The scavenging activity on hydroxyl radicals was expressed as:

$$\text{The scavenging activity on hydroxyl radicals} = \left[\frac{(A_0 - A_1)}{A_0 \times 100}\right]$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of extracts.

Data Analysis

Anti-inflammatory experimental data obtained from 'test' rats treated with ethanolic and aqueous extracts of *Moringa peregrina* and diclofenac, as well as those obtained from distilled water-treated 'control' rats, were pooled and expressed as means (±SD). The differences

between the plant's extracts and diclofenac-treated 'test' means, and distilled water-treated 'control' means, were analyzed statistically by one way analysis of variance (ANOVA; 95 % confidence interval), followed by Tukey-Kramer's multiple comparison test to assess the level of significance of the differences between the 'test' and 'control' group data means. Values of $p \leq 0.05$ were taken to imply statistical significance.

RESULTS

Injections of fresh egg albumin (0.5 ml/kg) provoked marked, time-related and progressive increases in the hind paw diameters of the 'control', untreated rats. Although pedal inflammation (oedema) was always evident within 5-8 min following fresh egg albumin (0.5 ml/kg) injection, maximal swelling and/or oedema occurred approximately 90 minutes following fresh egg albumin administration. Ethanolic and aqueous extracts of *Moringa peregrina* (100-300 mg/kg i. p.) also produced dose-related, significant reductions ($p < 0.05$-0.001) in the fresh egg albumin-induced acute inflammation of the rat hind paw (Table 1). Ethanolic and aqueous extracts (300 mg/kg) reduced inflammation by 81.01% and 72.69%, respectively at the third hour. A non-steroidal drug, diclofenac (100 mg/kg) exhibited anti-inflammatory effect with a maximum response 100% inhibition at the third hour. Distilled water (3 ml/kg i. p.) treatment alone has no anti-inflammatory effect on rat paw oedema induced by fresh egg albumin.

Figure 1 shows the reducing power of *Moringa peregrina* ethanolic and aqueous extracts. The reducing power of the two extracts increased with increasing concentration. Based on a comparison of the absorbance at 700 nm, the reducing power of ethanolic and aqueous extracts at a concentration of 20 μg/ml was similar to that of α-tocopherol at 20 μg/ml. This indicates that *Moringa peregrina* extracts was electron donors and could also react with free radicals, converting them to more stable products and terminate the radical chain reaction. Also, 140 μg/ml is the best concentration which exhibits the most reducing power. The reducing power of the two extracts and both standards decreased in the order of ethanolic extract > α-tocopherol >aqueous extract > BHA > BHT.

Figure 2a, b shows the chelating effect of ethanolic and aqueous extracts, respectively at 0.5, 1.0, 1.25 and 1.50 mg/ml concentration. The chelating activity of samples increased with increasing incubation times with $FeCl_2$. Also, the chelating activities of ethanolic and aqueous extracts at a concentration of 1.50 mg/ml are 60% and 37%, respectively at 90 min. In addition, no change in chelating activity of 1.50 mg/ml ethanolic extract with increasing the incubation times with $FeCl_2$ more than 90 min. However, the chelating activity of ethanolic extract of 1.0 mg/ml was nearly equal to EDTA

Table 1. Anti-inflammatory activity of ethanolic and aqueous extracts of *Moringa peregrina* (100-300 mg/kg i. p.) and diclofenac (100 mg/kg i. p.) on rat paw oedema induced by fresh egg albumin (0.5 ml/kg s. p.). Values quoted represent the mean (± SEM) of 8 observations. Percent inhibitions of the egg albumin-induced inflammation by the plant's extract and reference drug used are indicated as (%)

Experimental Group Dose (i.p.)	Time (in min) and paw diameter (in mm) 30 60 90 120				180
Control Group A (untreated)	10.45 ± 0.04	12.50 ± 0.05	15.65 ± 0.07	14.75 ± 0.06	11.35 ± 0.06
Control Group B (distilled water-treated) 3ml/kg	10.50 ± 0.035	12.46 ± 0.06	15.66 ± 0.04	14.80 ± 0.071	11.33 ± 0.04
Ethanolic extract (100 mg/kg)	9.82 ± 0.07*	10.98 ± 0.08*	12.18 ± 0.07*	9.26 ± 0.06*	6.53 ± 0.04**
Ethanolic extract (200 mg/kg)	9.20 ± 0.07*	9.64 ± 0.08*	9.12 ± 0.08*	8.00 ± 0.07**	5.12 ± 0.04**
Ethanolic extract (300 mg/kg)	5.40 ± 0.06**	4.98 ± 0.05**	3.66 ± 0.05***	2.80 ± 0.04***	2.80 ± 0.04***
Aqueous extract (100 mg/kg)	10.72 ± 0.08*	8.08 ± 0.08*	7.39 ± 0.05**	7.15 ± 0.05**	6.05 ± 0.06**
Aqueous extract (200 mg/kg)	8.10 ± 0.05*	7.42 ± 0.05**	7.15 ± 0.05**	5.72 ± 0.04**	4.05 ± 0.04***
Aqueous extract (300 mg/kg)	8.25 ± 0.22*	7.45 ± 0.15**	7.10 ± 0.08**	6.34 ± 0.06**	3.10 ± 0.03***
Diclofenac #	4.10 ± 0.06***	3.64 ± 0.05***	2.80 ± 0.04***	1.32 ± 0.03***	0.00 ± 0.00***

#Diclofenac is used as a reference

Experimental groups were compared to control group. Values are given as mean ± SD for groups of six animals each. *Significant at $P < 0.05$ **Significant at $P < 0.01$, ***Significant at $P < 0.001$

at 0.037 mg/ml (43.67%) for an incubation time of 10 min. This indicates that the chelating property of the samples on Fe^{2+} ions may afford protection against oxidative damage.

The DPPH· radical scavenging effects of ethanolic and aqueous extracts are presented in Figure 3 and showed appreciable free radical scavenging activities. The free radical scavenging activity of the extracts was

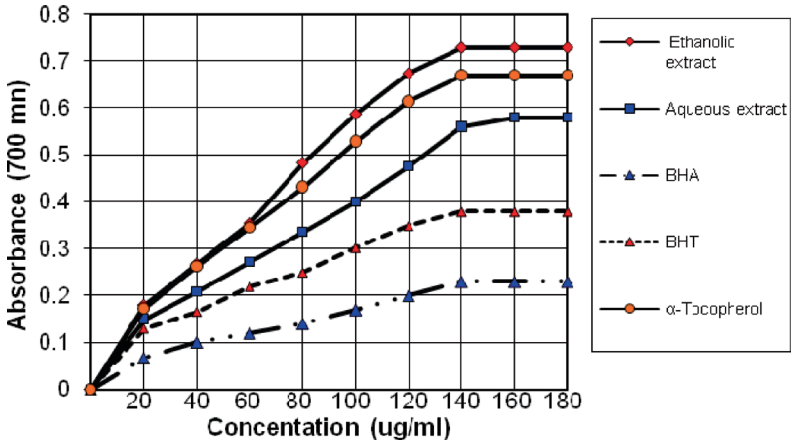


Figure 1. Reducing power of ethanolic and aqueous extracts, BHA, BHT and α-tocopherol.

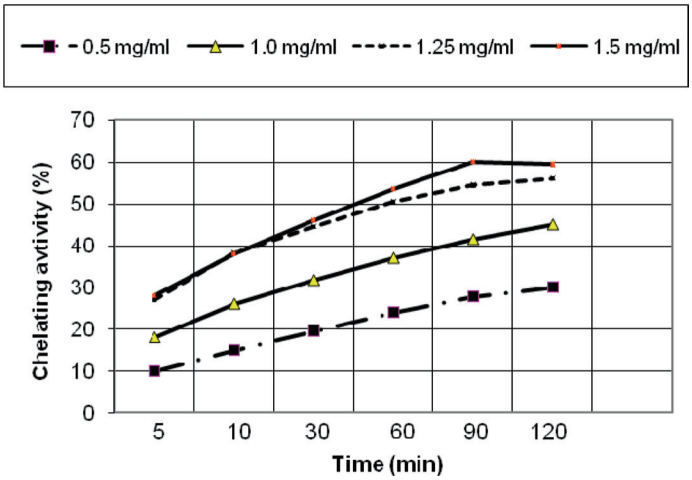


Figure 2a. Chelating effects of different concentrations of ethanolic extract on Fe^{2+} ions at different incubation times with $FeCl_2$.

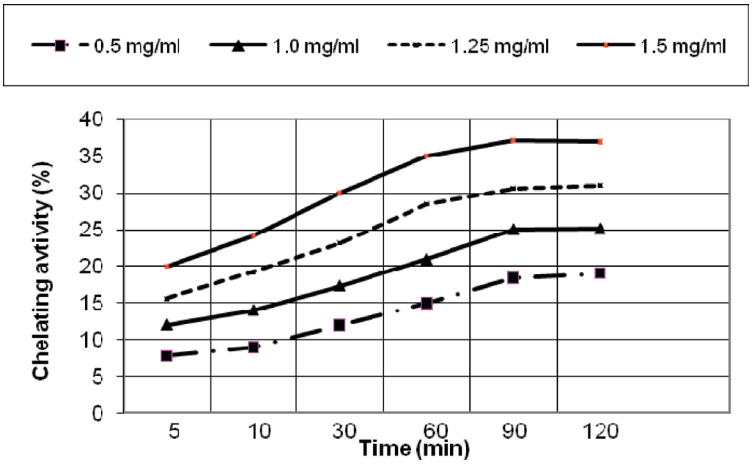


Figure 2b. Chelating effects of different concentrations of aqueous extract on Fe^{2+} ions at different incubation times with $FeCl_2$.

compared to trolox, as a synthetic antioxidant. Ethanolic extract of *Moringa peregrina* at 6 mg/ml had the highest radical scavenging activity when compared with 0.06 mg/ml trolox. But the radical scavenging activity of aqueous extract of 6 mg/ml was nearly equal to trolox at 0.06 mg/ml (72%).

The effects of 100 mg/l of ethanolic and aqueous extracts *Moringa peregrina* on peroxidation of linoleic acid emulsion are shown in Figure 4. The extracts showed higher antioxidant activity when compared to α-tocopherol, trolox, BHA, and BHT. Total antioxidant activity of *Moringa peregrina* extracts and both standards decreased in the order of ethanolic extract > α-tocopherol > aqueous extract > trolox > BHT> BHA. Also, the antioxidant activity of aqueous extract was nearly equal to α-tocopherol.

Figure 5 shows the superoxide radical scavenging activity of ethanolic and aqueous extracts *Moringa peregrina* at different concentrations 0.1, 0.5 and 1.0 mg/ml in comparison to the same amount of BHA, trolox and ascorbic acid. At 1.0 mg/ml concentration, ethanolic and aqueous extracts of *Moringa peregrina* showed higher superoxide radical scavenging activity than BHA and nearly equal to trolox and ascorbic acid. The superoxide radical scavenging activity of the two extracts and both standards decreased in the order of ascorbic acid > trolox > ethanolic extract > aqueous extract > BHA.

Figure 6 presents the scavenging activity of the extracts on H_2O_2. The results are compared with BHA, BHT, and α-tocopherol as standards. *Moringa peregrina* ethanolic and aqueous extracts were capable of scavenging activity in a concentration–dependent manner. At 100µg/ml, ethanolic and aqueous extract exhibited 79% and 65%, respectively. On the other hand, BHT, BHA, and α-tocopherol exhibited 28%, 35%, and 75%, respectively, of H_2O_2 scavenging activity at the same concentration.

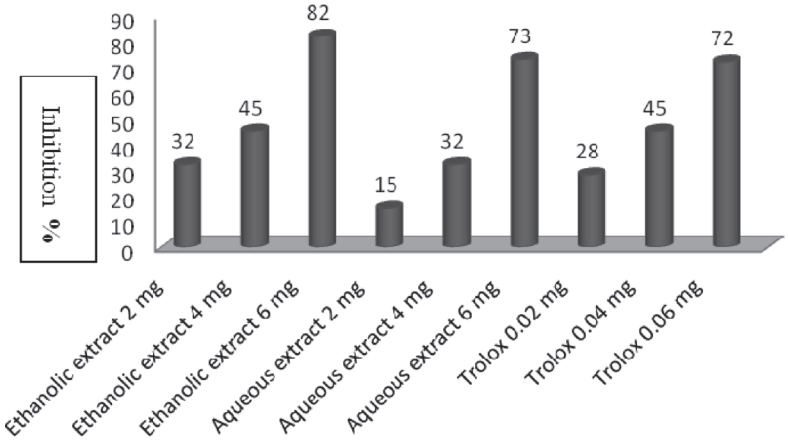


Figure 3. Scavenging activities of different concentrations of ethanolic and aqueous extracts and trolox against the 1,1-diphenyl-2-picryl-hydrazil (DPPH˙) radical.

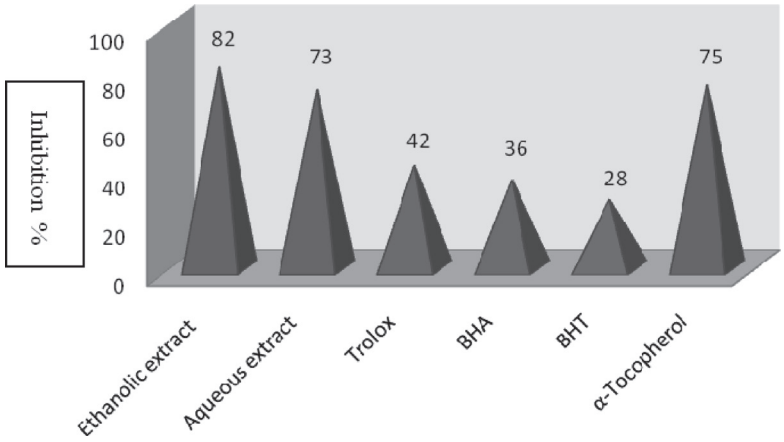


Figure 4. Total antioxidant activities of ethanolic and aqueous extracts, α-tocopherol, trolox and BHA, BHT (100 mg/l concentration) on peroxidation of linoleic acid emulsion.

Figure 7 shows the hydroxyl radical scavenging effects determined by the 2-deoxyribose oxidation method. The scavenging effect of *Moringa peregrina* ethanolic and aqueous extracts on hydroxyl radical was concentration dependent. At 20 and 40ug/ml the two extracts have the same hydroxyl radical scavenging activity. Also, at

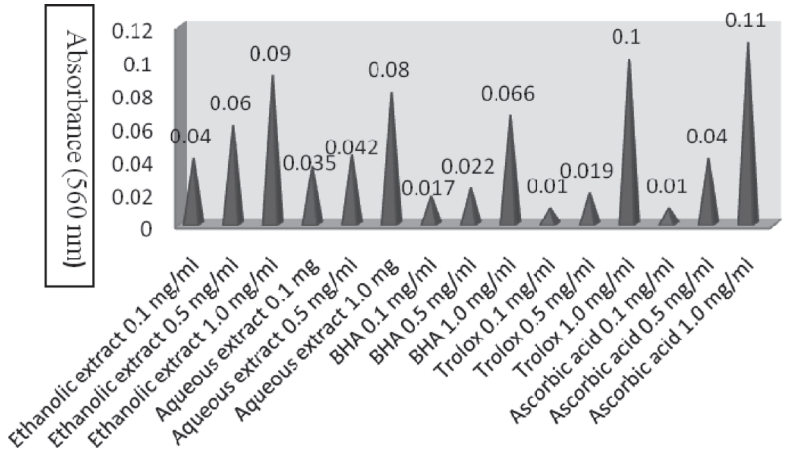


Figure 5. Superoxide radical scavenging activity of ethanolic and aqueous extracts, trolox, ascorbic acid and BHA at different concentrations.

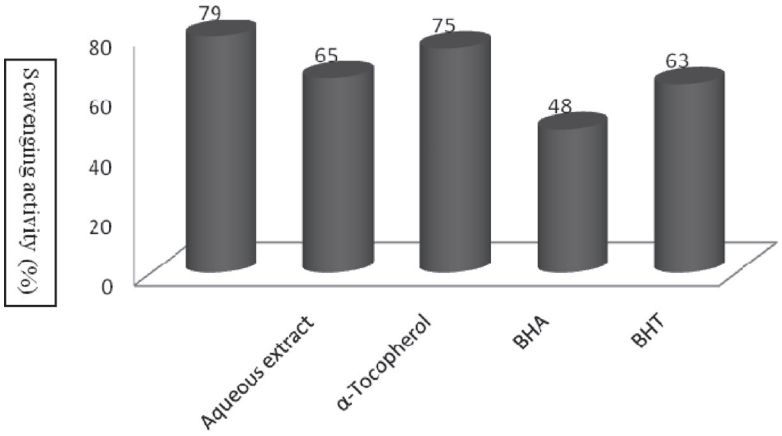


Figure 6. Hydrogen peroxide scavenging activity of ethanolic and aqueous extracts, α-tocopherol, BHT and BHA at 100µg/ml concentration.

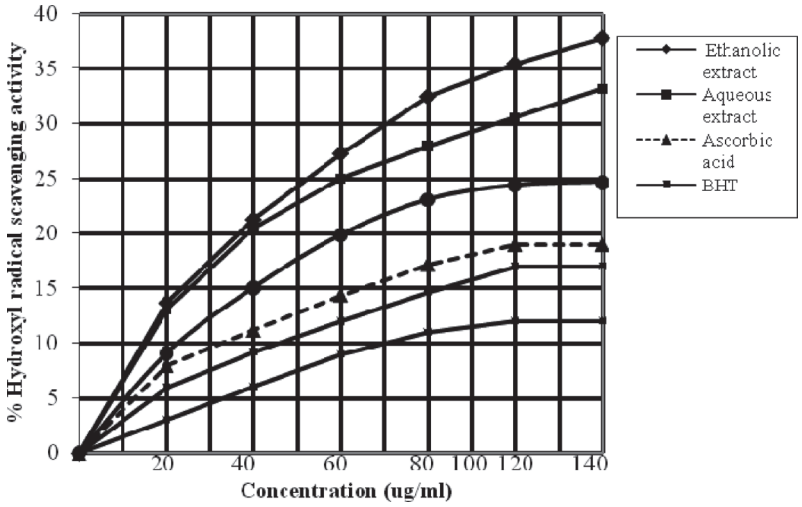
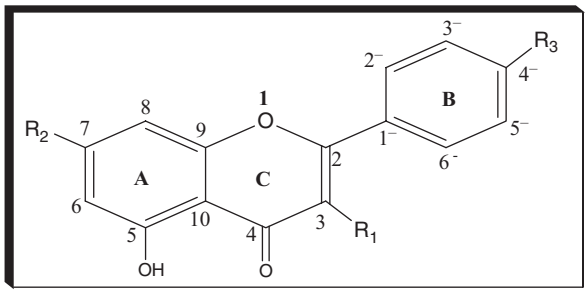


Figure 7. Hydroxyl radical scavenging activities of ethanolic and aqueous extracts, trolox, ascorbic acid, BHT and BHA at different concentrations.

20-140µg/ml concentrations, ethanolic extract exhibited higher hydroxyl radical scavenging activity than ascorbic acid. Among the oxygen radicals, hydroxyl radical is the most reactive chemical species known. The hydroxyl radical induces some oxidative damage to biomolecules such as all proteins, DNA, nucleic acid. The hydroxyl radical scavenging activity of the two extracts and both standards decreased in the order of ethanolic extract > aqueous extract > trolox > ascorbic acid > BHT> BHA.

DISCUSSION

Inflammation, which is a pattern of response to injury, involves the accumulation of cells and exudates in irritated tissues that allows protection from further damage. Inflammation has been studied for thousands of years in an attempt to combat its effects on the body. Experimental evidence obtained in the present study indicates that the ethanolic and aqueous extracts of *Moringa peregrina* (100-300 mg/kg i.p.) dose-dependently and significantly delayed ($p < 0.05$-0.001) the inflammation. Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is commonly employed in the treatment and/or management of rheumatoid arthritis, osteo-arthritis and ankylosing spondylitis[38] and for its anti-inflammatory and analgesic effects.[39] Diclofenac reduces inflammation, swelling and arthritic pain by inhibiting prostaglandins synthesis and/or production.[40] The drug also affects polymorphonuclear leukocytes function *in vitro*, thereby reducing chemotaxis, superoxide toxic radical formation, oxygen-derived free radical generation and neutral protease production.[41] Diclofenac has also been reported to suppress inflammation induced by various phlogistic agents in experimental animal models.[42] Although the anti-inflammatory and antioxidant effect of ethanolic and aqueous extracts of *Moringa peregrina* could not be discussed in any article before that and our study is the first type in this kind, the results obtained suggest that *Moringa peregrina* extracts may produce its anti-inflammatory effect due to the presence of tannins,[43] flavonoids[44] (figure 8), saponins,[45] unsaturated sterols and/or triterpenes[15] figure 9. *In vivo* and *in vitro* anti-inflammatory effects have been reported for several flavonoids. Thus, some flavones have been found to be active in different experimental models. B-ring substituted flavones of this type are capable of inhibiting cotton-pellet induced granuloma and carrageenan edema in rats and TPA-mouse ear edema.[46] Nevertheless, the influence of *Moringa peregrina* extracts on the inhibition of inflammation is not yet completely understood.



Compound 1: **kaempferol 3-O-(2″,3″-diacetylglucoside)**,

R_1 = *O*-2″,3″-Diacetylglucoside, R_2 = OH, R_3 = OCH_3

Compound 2: **kaempferol 3-O-(2″-O-galloylrhamnoside)**,

R_1 = *O*-[Glucosyl-(1‴ → 2″)]-[rhamnosyl (1⁗ → 6″)-glucoside, R_2 = *O*-rhamnose, R_3 = OH

Compound 3: **kaempferol 3-O-(2″-O-galloylrutinoside) -7-O- α-rhamnoside**,

R_1 = *O*-(2″-Galloylrhamnoside), R_2 = OH, R_3 = OCH_3

Figure 8. structure of flavonoid derivatives from *Moringa*(11)

Saponins are a group of glycosides found in many plants. Saponins can be classified into two groups based on the nature of their aglycone skeleton. One group consists of the steroidal saponins and the other group consists of the triterpenoid saponins.[47] There are a number of saponins isolated from various plants which have anti-inflammatory activity.[48] The presence of saponins in *Moringa peregrina* extracts may produce its anti-inflammatory effect.

II. Evaluation of Antioxidant Property

1. Determination of reducing power: The effects of free radicals on human beings are closely related to toxicity, disease and aging (49). Most living species have an efficient defense system to protect themselves against the oxidative stress induced by Reactive Oxygen Species (ROS).[50] Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases.[5-8] Human organism possesses systems controlling oxidation processes posing a threat to structures and functions of cells. Three defense mechanisms has been developed,[51] including: prevention of reactions of reactive oxygen species with biologically-significant compounds, breaking free-radical chain reactions and undesirable non-radical oxidation reactions, scavenging the products of free radicals reactions with biological substances and repair of damages. Flavonoid's activity as antioxidants refers to their ability to transfer a hydrogen atom or an electron and to the possibility of their interactions with other

antioxidants.[52] The reducing power has been used as one of the antioxidant capability indicators of plants.[53] In the reducing power assay, the presence of reductants (antioxidants) in the tested extracts resulted in the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) figure 1. The amount of Fe^{2+} complex can therefore be monitored by measuring the formation of Perl's Prussian Blue at 700 nm.[1]

The results in figure 1 the reducing power of ethanolic and aqueous extracts from *Moringa peregrina*. The reducing power of all the extracts increased with increasing concentration. Based on a comparison of the absorbance at 700 nm, the reducing power of *Moringa peregrina* extracts at a concentration of 20µg/ml was similar to that of α-tocopherol at 20µg/ml. This indicates that the extracts of *Moringa peregrina* were electron donors and could also react with free radicals, converting them to more stable products and terminate the radical chain reaction.

The presence of flavonoids in *Moringa peregrina* extracts the extracts, which have been reported to possess several biological properties. Despite the similarity between flavonoid structures, the biological properties vary considerably with only minor modifications in their structure. The number and specific positions of hydroxyl groups and the nature of the substitutions determine whether flavonoids function as strong antioxidative,[54] anti-inflammatory, antiproliferative[55] or enzyme modulating agents. Hydroxylation of the B-ring, where a catechol group is the key to the flavonoid activity, coupled with a 2, 3 double bond in conjugation with a 4-oxo function (carbonyl group) in the C-ring.[54]

The antioxidant properties of *Moringa peregrina* flavonoids resulted from their chemical structure: 4'-hydroxyl system in the B ring, reciprocal configuration of the double bond C2-C3 and the 4-carbonyl group of the C ring, and configuration of the 5-hydroxyl group of the A ring[56] (Figure 8). All the mentioned structural conditions may be found in *Moringa peregrina* flavonoid; kaempferide flavonoids which, in the *in vitro* systems efficiently scavenges hydroxyl radical ($OH^{\bullet}$), superoxide radical ($LOO^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and nitrogen oxide ($NO^{\bullet}$).

2. Determination of chelating activity on Fe^{2+}: Iron is the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron can stimulate lipid peroxidation by the Fenton type reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The Fe^{3+} ion also produces radicals from peroxides, although the rate is ten times less than that of the Fe^{2+} ion. The Fe^{2+} ion is the most powerful pro-oxidant among the various species of metal ions.[57] Ferrozine, a chelating reagent, was used to indicate the presence of chelator in the reaction system. Ferrozine forms a complex with free Fe^{2+} but not with Fe^{2+} bound by extracts. In the presence of chelating agents, the complex formation of ferrous iron and ferrozine is disrupted, resulting in a decrease in the red color of the complex. Measurement of color reduction therefore allows the metal chelating activity of the coexisting chelator to be estimated.[58] The purpose of the test of ferrous ion chelating activity was to determine the capacity of *Moringa peregrina* extracts to bind the ferrous ion catalyzing oxidation. The ferrous ion chelating effect of *Moringa peregrina* extracts is presented in Figure 2a-b. The ethanolic and aqueous extracts at 1.5 mg/ml concentration showed 55% and 35% chelating effect on ferrous ions at an incubation time of 60 min., respectively. The chelating activity of samples increased with increasing incubation times with $FeCl_2$. At the same amounts, ethanolic exhibited higher chelating activity on Fe^{2+} than aqueous extracts. However, the chelating activity of ethanolic extract of 1.0 mg/ml was nearly equal to EDTA at 0.037 mg/ml (43.67%) for an incubation time of 90 min. This indicates that the chelation property of the samples on Fe^{2+} ions may afford protection against oxidative damage.

3. Determination of free radical-scavenging activity: Free radicals are known to be a major factor in biological damages, and $DPPH^{\bullet}$ has been used to evaluate the free radical-scavenging activity of natural antioxidants.[26] $DPPH^{\bullet}$, which is a molecule containing a stable free radical with a purple color, changes into a stable compound with a yellow color by reacting with an antioxidant which can donate an electron to $DPPH^{\bullet}$, Figure 3. In such case, the purple color typical of the free $DPPH^{\bullet}$ radical decays, a change which can be followed either spectrophotometrically (517 nm). The proton radical scavenging action is known as an important mechanism of antioxidation. 1,1-Diphenyl-2-picrylhydrazil ($DPPH^{\bullet}$) is used as a free radical to evaluate the antioxidative activity of some natural sources.[1] From these results, it can be stated that *Moringa peregrina* extracts have the ability to scavenge free radicals and could serve as a strong free radical inhibitor or scavenger according

to trolox. On the other hand, *Moringa peregrina* flavonoids may be particularly important in protecting cellular DNA, lipids and proteins from free radical damage. It has been reported that the antioxidant activity of flavonoids may result from the neutralization of free radicals initiating oxidation processes, or from the termination of radical chain reactions, due to their hydrogen donating ability.[59] It is also known that the antioxidant activity of polyphenolic compounds is closely associated with their structures, such as substitutions on the aromatic ring and side chain structure. Their accessibility to the radical centre of DPPH• could also influence the order of the antioxidant power. Free radical scavenging activity of polyphenolic compounds is believed to be influenced by the number and position of phenolic hydrogen in their molecules.[54] It is also proposed that the higher antioxidant activity is related to the greater number of hydroxyl groups on the flavonoid nucleus.[60]

4. Determination of total antioxidant activity determination: The total antioxidant activity of *Moringa peregrina* extracts was determined by the thiocyanate method in linoleic acid emulsion. The antioxidative activities of *Moringa peregrina* ethanolic and aqueous extracts were compared with commercial antioxidants such as α-tocopherol (Toc), BHT, BHA and trolox. Total antioxidant activity of *Moringa peregrina* extracts and both standards decreased in the order of ethanolic extract > α-tocopherol > aqueous extracts > trolox > BHA > BHT figure 4.

5. Determination of superoxide radical scavenging activity: Superoxide radical, known to be very harmful to cellular components as a precursor of the more reactive oxygen species, contributes to tissue damage and various diseases. In a biological system, its toxic role can be eliminated by superoxide dismutase.[1] The radicals may also play an important role during the peroxidation of unsaturated fatty acids and other potential susceptible substances.[61] The superoxide radical scavenging activity of *Moringa peregrina* extracts and both standards decreased in the order of ascorbic acid > trolox > ethanolic extract > aqueous extracts > BHA figure 5. In the terms of molecules of superoxide radical, *Moringa peregrina* extracts were equivalent to ascorbic acid. The reaction mechanism of superoxide radical with superoxide radical can be explained by two-steps reaction: in the first step, one molecule of superoxide radical reacts with one molecule of ascorbic acid produce ascorboxy radical and oxide radical. Then, one ascorboxy radical reacts with another molecule of oxide radical to form ascorboxyl-quinone. Hence, one molecules of superoxide radical are reduced by one molecule of ascorbic acid.

6. Determination of hydrogen peroxide scavenging activity: Hydrogen peroxide is an intermediate during endogenous oxidative metabolism and mediates radical oxygen formation such as OH, which may be used to predict the scavenging capability of antioxidants in biological systems.[32] H_2O_2 has only a weak activity to initiate lipid peroxidation, but its activity as an active oxygen species comes from its potential to produce the highly reactive hydroxyl radical through the Fenton reaction. These results in figure 6 showed that *Moringa peregrina* extracts have a strong H_2O_2 scavenging activity. At 100 μg/ml concentration, H_2O_2 scavenging activity of extracts and both standards decreased in the order of ethanolic extract > α-tocopherol > aqueous extract > BHT > BHA. The mechanism of *Moringa peregrina* extracts antioxidant activity was designed by hydrogen donation to free radicals and formation of a complex between the lipid radical and the antioxidant radical (*Moringa peregrina* polyphenols, free radical acceptor). In the terms of molecules of hydrogen peroxide, resveratrol was equivalent to α-tocopherol. The reaction mechanism of hydrogen peroxide with α-tocopherol can be explained by two-steps reaction: in the first step, one molecule of hydrogen peroxide reacts with one molecule of α-tocopherol produce α-tocopheroxy radical and $^{-}$OH. Then, one α-tocopheroxy radical reacts with another molecule of hydroxyl radical to form α-tocopherolquinone. Hence, one molecules of hydrogen peroxide are reduced by one molecule of α-tocopherol.

7. Determination of hydroxyl radical scavenging activity: The scavenging effect of ethanolic and aqueous extracts of *Moringa peregrina* on hydroxyl radical was concentration dependent. At 20-140 μg/ml concentrations, ethanolic and aqueous extracts of *Moringa peregrina* exhibited lower hydroxyl radical scavenging activity than ascorbic acid. At 20 and 40 μg/ml concentrations, ethanolic and aqueous extracts of *Moringa peregrina* showed the same hydroxyl radical scavenging activity. At 60-140 μg/ml concentrations, ethanolic extract of *Moringa peregrina* showed higher hydroxyl radical scavenging activity than aqueous.

The antioxidant capacity of the *Moringa peregrina* extracts is mainly dependent on phenolic compounds.

The presence of phenolics constitute; tannins,[43] flavonoids[44] figure 8, saponins,[45] unsaturated sterols and/or triterpenes[15] figure 9, all of these major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the *Moringa peregrina* plant extracts. In this study, the phenols contain hydroxyls that are responsible for the radical scavenging effect mainly due to redox properties.[62] According to our study, the high phenolic content in of *Moringa peregrina* can explain its high free radical scavenging activity.

The anti-inflammatory and antioxidant activities of *Moringa peregrina* seeds ethanolic and aqueous extracts have not been reported earlier to our knowledge and this study is might be the first observation of that kind.

In conclusion, the methods selected to assess the antioxidant activity of the *Moringa peregrina* ethanolic and aqueous extracts roughly represented different systems like reducing power, chelating activity on Fe^{2+}, free radical-scavenging, total antioxidant, superoxide radical scavenging, hydrogen peroxide scavenging and hydroxyl radical scavenging activities. The present study showed that the effects of antioxidative activity of *Moringa peregrina* extracts depend on the presence of bioactive polyphenolics constitute; tannins, flavonoids, saponins, unsaturated sterols and/or triterpenes.

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