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In vitro Antioxidant Activity, Phytochemical analysis and Cytotoxicity of *Ethanolic* leaves Extract of *Moringa oleifera* lam

[1] Mohammed A Abass, [1] Ahmed S Kabbashi, [2] Mohammed I Garbi

[1] Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), P.O. Box 2404, National Center for Research, Khartoum, Sudan.

[2] Department of Microbiology, Faculty of Medical Laboratory Sciences, International University of Africa. P.O. Box 2469 Khartoum, Sudan.

Abstract

Interest in natural products as a source for innovation in drug discovery and agrochemicals is still growing worldwide. Natural products, whose immense diversity has been appreciated for many years, may become in a rich source of novel chemical structures. The present study was conducted to investigate the *in-vitro* antioxidant (DPPH assay), phytochemical screening and cytotoxicity (MTT-assay) of ethanol extract of *Moringa oleifera* (leaves). The methanol extract of *M. oleifera* (leaves) was tested for antioxidant screening for their free radical scavenging properties using 2.2Di (4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH), while propyl galate was used as standard antioxidant, phytochemical screening and cytotoxicity(MTT assay) with different concentration (500 ppm, 250 ppm and 125 ppm) and compare triton-100 (the reference control). The ethanol extract of *M. oleifera* (leaves) antioxidant activity was (75 ± 0.01 RSA%) in comparison to the control of propyl galate levels (88 ± 0.07RSA%), Preliminary phytochemical screening of the leaves of *M. oleifera* revealed that the plant contain triterpenes, alkaloids, tannins, glycosides, coumarins saponins and flavonoids. Negative results were recorded for alkaloids. And in addition cytotoxicity (MTT assay) with different concentration (500, 250 and 125 ppm) in comparison to triton-x100 (the reference control) which verified the safety of the examined extract with an IC50 less 100 µg/ml. Hence, the results obtained in the present study indicate that *M. oleifera* have promising antioxidant indicates that the plant could be promising agent in scavenging free radicals and treating diseases related to free radical reactions.

Keywords: *In vitro*, antioxidant activity, cytotoxicity (MTT-assay), *Moringa oleifera* (leaves), Sudan.

Introduction

Reactive oxygen species (ROS) have been implicated in the induction of various types of oxidative damage to biomolecules that results against, cancer, neurodegenerative diseases, atherosclerosis, malaria, several pathological events in living organisms and different other diseases associated with our life-style (Shahidi and Naczk, 1995; Halliwell *et al*., 1992). These molecules can induce changes in different biological tissues and cell biomolecules such as lipids, proteins, DNA or RNA. Free radicals can also affect food quality; reducing its nutritional content and promoting the development of food deterioration (Nickavar and Aboalhasani, 2009).

Recently in many African countries comprehensive research was conducted on medicinal plants for the treatment of different diseases and conditions, such as diabetes, malaria, anaemia, and cancer. The availability and relatively cheaper cost of medicinal plants in sub-Saharan Africa, makes them more attractive as therapeutic agents when compared to 'modern' medicines (Agbor *et al*., 2005).

The medicinal properties of plants have been investigated, in t he light of recent scientific developments, through out the wor ld due to their potent pharmacological activities and economic viability. A great number of aromatic, spicy, me dicinal and other plants contain chemical compounds, exhibiti ng antioxidant properties. Source of natural antioxidants are pr imarily, plant phenolics that may occur inall parts of plants suc h as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt, 1990). Many of these antioxidantcompounds possess anti-inflammatory, antiatherosclerotic, antitumor, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent (Sala *et al*, 2002). Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolic are increasingly of interest in the food industry, because they retar d oxidative degradation of lipids and thereby improve the qual ity and nutritive value of food (Kahkonen *et al*, 1999; Rice *et al*, 1995).

Moringa oleifera Lam. is the most widely cultivated species of a monogeneric (family: Moringaceae) that is native to the sub-Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan (Fahey, 2005) which is widely used for treating bacterial infection, fungal infection, antinflammation, sexually-transmitted diseases, malnutrition and diarrhoea. Moringa species have long been recognized by folk medicine practitioners as having value in the treatment of tumors (Ramachandran *et al*., 1980). Gupta *et al*., (2005) reported chelating property against arsenic toxicity. Gupta *et al*., (2005) A total of forty four compounds were isolated from the leaves of *Moringa oleifera* (Lam.). Chuang *et al*., (2007) and four pure compounds were also isolated which showed a blood pressure lowering effect. Gilani *et al*., (1994) Moringine and Moringinine were the two alkaloids isolated from the stem bark of *Moringa oleifera* (Lam.). Kerharo, (1996) Methanol extract of *Moringa oleifera* (Lam.) root showed central nervous system depressant action was reported by Gupta *et al*., (1999). The

aqueous extract of *Moringa oleifera* (Lam.) root possess antifertility property. Shukla *et al*., (1998) *Moringa oleifera* (Lam.) seed also exhibited cyanobacteriacidal activity. Lurling and Beekman (2010) *Moringa oleifera* (Lam.) leaves possess hypolipidaemic and antiathersclerotic activities. Pilaiprk *et al*., (2008) *Moringa oleifera* (Lam.) seed has excellent coagulation properties for treating waste water proved by Ndabigengesere *et al*., (1995). *Moringa oleifera* (Lam.) pods was studied for free radicals scavenging abilities Kumar *et al*., (2007). Leaves of *Moringa oleifera* Purgative, applied as poultice to sores, rubbed on the temples for headaches, used for piles, fevers, sore throat, bronchitis, eye and ear infections, scurvy and catarrh; leaf juice is believed to control glucose levels, applied to reduce glandular swelling (Morton, 1991; Makonnen *et al*.,1997; The Wealth of India, 1962; Dahot, 1988). The present study was conducted to investigate the antioxidant activities, phytochemical screening and cytotoxicity of *M. oleifera* (leaves) in Sudan.

II. Materials and Methods

Collection of *Moringa oleifera*

Moringa oleifera (leaves) were collected from Khartoum state, farm (June 2014), and authenticated by the researcher **Dr. Haider Abdelgadir**, Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI).

Preparation of *Moringa oleifera* leaves

M. oleifera leaves samples were dried in the shade then milled using Mortar and Pestle to give powder.

Preparation of crude extracts

Extraction was carried out for the leaves of *M. oleifera* by using overnight maceration techniques according to the method described in Harbone (1984). About 50 g round material was macerated in 250 ml of ethanol for 3 h at room temperature. Occasional shaking for 24 h at room temperature was performed and, the supernatant was decanted. Thereafter, the supernatant was filtered under reduced pressure by rotary evaporatorion at 55 °C. Each residue was weighed and the yield percentage was calculated and then stored at 4 °C in tightly sealed glass vial ready for use. The remaining extracts which were not soluble were successively extracted using ethanol with the described technique. The extracts were kept in freez dryer for 48 h, (Virtis, USA) until they were completely dried. The residue was weighed and the yield percentage was calculated. The extracts were kept and stored at 4°C until required.

Antioxidant activity of *M. oleifera* extracts

DPPH radical scavenging assay

The DPPH radical scavenging was determined according to the method by (Shimada *et al*., 1992) with some modification. In 96-wells plate, the test samples were allowed to react with 2.2Di (4-tert-octylphenyl)-1-picryl hydrazyl stable free radical (DPPH) for half an hour at 37 °C. The concentration of DPPH was kept as 300 µm.The extract was dissolved in DMSO (500µg/ml.concentration), while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517 nm using multiplate reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group and propyl gallate (PG). All tests and analysis were run in triplicate.

Phytochemical Screening

Phytochemical screening is of great importance in providing us with information about chemicals found in the plant in term of their nature and range of occurrence. This information would enable us to correlate between the nature and range of occurrence of these chemicals and biological assays held to investigate a certain bioactivity of the mentioned plant. In this study the preliminary phytochemical screening was conducted according to (Haborne, 1984).

Preparation of the Extracts

10 mg of the powdered leaves of each plant were refluxed with 100 ml of ethanol 80% for 4 hours. The cool solution was filtered and enough ethanol 80% was passed through the volume of the filtrate 100 ml. This prepared extract (PE) was used for the various tests.

Test for Unsaturated Sterols and Triterpenes

10 ml of the prepared extract (PE) was evaporated to dryness on a water bath and the cooled residue was stirred several times with petroleum ether to remove most of the coloring materials. The residue was then extracted with 20 ml of chloroform. The chloroform solution was dehydrated over sodium sulphate anhydrous. 5 ml of chloroform solution was mixed with 0.5 ml acetic anhydride followed by two drops of conc. Sulphuric acid. The gradual appearance of green, blue pink to purple color was taken an evidence of the presence of sterol (green to blue) and or triterpenes (pink to purple) in the sample (Harborne, 1984).

Test for Alkaloids

7.5 ml of (PE) was evaporated to dryness on a water bath. 5 ml of HCl (2N) was added and stirred while heating on the water bath for 10 minutes, cooled filtered and divided into two test tubes. To one test tube few drops of Mayer's reagent were added. While to the other tube few drops of Valser's reagent were added. A slight turbidity or heavy precipitate in either of the two test tubes was tanked as presumptive evidence for the presence of alkaloids (Harborne, 1984).

Test for Flavonoids

17.5 ml of the (PE) was evaporated to dryness on a water bath, cooled and the residue was defatted with petroleum ether and the defatted residue was dissolved in 30 ml of ethanol (80%) and filtered. The filtrate was used for the following tests: (A) To 3 ml of the filtrate in a test tube 1 ml of 1% aluminum chloride solution was in methanol was added. Formation of yellow color indicated the presence of Flavonoids, (Flavones and / or chalcone). (B) To 3 ml of the filtrate in a test tube 1 ml of 1% potassium hydroxide solution was added. A dark yellow color indicated the presence of the Flavonoids compounds (flavones or flavanones) chalcone and/or flavonol. (C) To 2 ml of the filtrate 0.5 ml of magnesium turnings were added. Producing of defiant color to pink or red was taken as presumptive evidence that flavanones were present in the plant sample (Harborne, 1984).

Test for Tannins

7 ml of the (PE) was evaporated to the dryness on water bath. The residue was extracted several times with n-hexane and filtered. The insoluble residue was stirred with 10 ml of saline solution. The mixture was cooled, filtered and the volume of the

filtrate was adjusted to 10 ml with more saline solution. 5 ml of this solution was treated with few drops of gelatin salt reagent. Formation of immediately precipitate was taken as an evidence for the presence of tannin in plant sample. To another portion of this solution, few drops of ferric chloride test reagent were added. The formation of blue, black or green was taken as an evidence for the presence of tannins.

Test for Saponins

1 g of the original dried powdered plant material was placed in a clean test tube. 10 ml of distilled water was added and the tube was stoppered and vigorously shaken for about 30 seconds. The tube was then allowed to stand and observed for the formation of (honeycomb). The appearance of honeycomb, which persisted for least an hour, was taken as an evidence for the presence of Saponins.

Test for Anthraquinone Glycosides

10 g of the powdered plant sample were boiled with 10 ml of 0.5N KOH containing 1 ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with10 ml of benzene. 5 ml of the benzene solution was shaken with 3 ml of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of Anthraquinones was indicated if the alkaline was found to have assumed pink or red color.

Test for Coumarins

3 g of the original powdered plant sample was boiled with 20 ml of distilled water in a test tube and filter paper was attached to the test tube to be saturated with the vapor after a spot of 0.5N KOH put on it. Then the filter paper was inspected under UV light, the presence of coumarins was indicated if the spot has found to be absorbed the UV light.

Cytotoxicity Screening

Microculture tetrazolium MTT assay was utilized to evaluate the cytotoxicity of the *M. oleifera***.**

Microculture Tetrazolium (MTT) Assay
Principle

This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, blue colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells (Patel, *et al*., 2009).

Preparation of *M. oleifera* extract

Using a sensitive balance 5 mg of each extracts were weighed and put in eppendorf tubes. 50 µl of DMSO were added to the extract and the volume was completed to 1 ml with distilled water obtaining a concentration of 5 mg/ml. The mixture was vortexed and stirred by magnetic stirrer to obtain a homogenous solution.

Cell Line and Culturing Medium

Vero (Normal, African green monkey kidney) cells were cultured in a culturing flask containing a complete medium consisting of 10% fetal bovine serum and 90% minimal essential medium (MEM) and then incubated at 37ºC. The cells were sub cultured twice a week.

Cell line used

Vero cells (Normal, African green monkey kidney).

Cell counting

Cell counts were done using the improved Neubauer chamber. The cover slip and chamber were cleaned with detergent, rinsed thoroughly with distilled water and swapped with 70% ethanol, then dried. An aliquot of cell suspension was mixed with equal volume of 0.4% trypan blue in a small tube. The chamber was charged with cell suspension. After cells had settled, the chamber was placed under light microscope. Using 40 X objective, cells in the 4 large corner squares (each containing 16 small squares) were counted. The following formula was used for calculating cells:

$$\textbf{(Cells/ml) N} = \frac{\textbf{Number of cells counted X Dilution factor X } 10^4}{4}$$

Procedure

The monolayer cell culture formed in the culturing flasks was trypsinized and the cells were put in centrifuging tube and centrifuged for 5 minutes separating the cells from the supernatant that flicked out. 1 ml complete medium was added to the cells and all the cell suspension was contained in a basin. In a 96- well microtitre plate, serial dilutions of each extracts were prepared. 3 duplicated concentrations for each extracts i.e. 6 wells for each of 8 extracts. All wells in rows A, B and C were used in addition to first 4 wells from each rows D, E and F. The first 2 wells of row G were used for the negative control and the first 2 wells of row H were used for the positive control Triton X. 20 µl complete medium pipetted in all wells in rows B, C and mentioned wells of rows E and F. Then 20 µl from each extracts were pipetted in rows A and B and first 4 wells of rows E and F. 20 µl taken from row B were pipetted and mixed well in row C from which 20 µl were taken and flicked out. The same was done from E to F. After that 80 µl complete medium were added to all used wells. Then adjusting the cell account to 3000 cell/well, 100 µl of cell suspension were added completing all wells to the volume 200 µl. Now, we have duplicated three concentrations 500, 250, 125 µg/ml for each extract. Then the plate was covered and incubated at 37ºC for 96 hours.

On the fourth day, the supernatant was removed from each well without detaching the cells. MTT suspension stock (5 mg/ml) prepared earlier in 100 ml phosphate buffer solution (PBS) was diluted (1:3.5) in a culture medium. To each well of the 96-well plate, 50 µl of diluted MTT were added. The plate was incubated for further 4 hours at 37ºC. MTT was removed carefully without detaching cells, and 100 µl of DMSO were added to each well. The plate was agitated at room temperature for 10 minutes then read at 540 nm using microplate reader. The percentage growth inhibition was calculated using the formula below:

$$\textbf{\% Cell inhibition} = 100-\{(Ac-At)/Ac\} \times 100$$

Where, **At**= Absorbance value of test compound;
Ac= Absorbance value of control.

Statistical analysis

All data were presented as means ± S.D. Statistical analysis for all the assay results were done using Microsoft Excel program (2007).

III. Results and Discussion

Ethanolic leaves extract of *M. oleifera* (family: Moringaceae) were screened for antioxidant screening for their free radical scavenging properties using 2.2Di (4-tert-octylphenyl)-1-picryl-hydrazyl (DPPH), while propyl galate was used as standard antioxidant and phytochemical screening, and cytotoxicity (MTT assay) with different concentration (500 ppm, 250 ppm and 125 ppm) and compare triton-100 (the reference control).

Antioxidant activity of *M. oleifera* (leaves) extract:

This table indicate the anti DPPH of ethanol extract of *M. oleifera* (leaves), propyl gallate was used as standard drug level. The tested antioxidant activity gave (75 ± 0.04 RSA %) in comparison to the control of propylgalate levels gave (88 ± 0.07 RSA %).

As shown in Table (1), the results of antioxidant activity *M. oleifera* (leaves) showed high antioxidant activity against the DPPH free radical (81.04 RSA%). This result was similar to that reported by Chumark *et al.* (2008) using free radical scavenging activity of Moringa oleifera using 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH).

Table 1: Antioxidant activity of *M. oleifera* (leaves)

No	Name of plant	Part	%RSA* ± SD (DPPH)
1	*M. oleifera*	leaves	75 ± 0.01
2	*Control	PG	88 ± 0.07

Key: RSA* = Radicals scavenging activity
***Control = P.G** = Propyl Gallate.

Phytochemical analysis of *M. oleifera* (leaves)

The Phytochemical analysis of crude ethanolic extract of *M. oleifera* performed by the method described earlier and then and then analyzed for phytocompounds like steroids or terpenoids, alkaloids, flavonoids, coumarins, saponins, tannins and anthraquinone preliminary analyzed and present in the (Table 2).

The Phytochemical analysis of crude ethanolic extract of *M. oleifera* performed by the method described earlier and then and then analyzed for phytocompounds like steroids or terpenoids, alkaloids, flavonoids, coumarins, saponins, tannins and anthraquinone preliminary analyzed and present study. The Alkaloids is absent in both the case of plant extracts. This result was similar to that reported by Rajamanickam and Sudha, (2013).

Table 2: Preliminary Phytochemical Screening analysis of *M. oleifera* (leaves) extract

No.	Tested	*M. oleifera* (leaves)
1	Unsaturated Sterol And/or Triterpenes	+
2	Alkaloids	-
3	Flavonoids	+
4	Tannins	+
5	Saponins	+
6	Anthraquinone glycoside	+
7	Coumarins	+

\+ = Present = Absent.

Cytotoxicity assay of *M. oleifera* (leaves) extract:

Interestingly, the cytotoxicity assays were conducted in this study to evaluate the cytotoxicity effects of ethanolic extract of *M. oleifera* (leaves) by using MTT-assay including (Vero cell line). Table 3 indicated the inhibition percentage (%) of Vero cell line growth *in vitro* by ethanolic extract of *M. oleifera* (leaves) for different concentrations 125 to 500 µg/ml and showed an IC_{50} >100 (µg/ml) which is verifying the plant safety. The maximum concentration used was 500 µg/mL. When this concentration produced less than 50% inhibition, the IC_{50} cannot be calculated.

Table 3: Cytotoxicity of *M. oleifera* (leaves) extract on normal cell lines (Vero cell line) as measured by the MTT assay

No.	Name of samples (part)	Concentrations (µg/ml)	Absorbance	Inhibition (%) ± SD	IC_{50} (µg/ml)
1	*M. oleifera* (leaves)	500	2.42	17.9 ± 0.05	**100>**
		250	2.87	1.8 ± 0.03	
		125	3.42	-17.5 ± 0.02	
2	*Control		0.14	95.3 ± 0.01	

Key: *Control = Triton-x100 was used as the control positive at 0.2 µg/mL.

V. Conclusion

From complete investigation about antioxidant and pharmaceutical screening studies of *M. oleifera* leaves it can be recommended that extracts could be used as a easily available foundation of natural antioxidants, which can be used as supplement to aid the therapy of free radical mediated diseases such as cancer, diabetes, inflammation, etc., diabetes swelling. Further studies are needed on the isolation and elucidation of their chemical structures of antioxidant components, and other related pharmacological studies such as *in vivo* investigation, drug formulation and clinical trials are highly recommended.

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