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Antibacterial and haematological activity of *Moringa oleifera* aqueous seed extract in Wistar albino rats

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Abstract

The phytochemical, antibacterial and haematologic activity of aqueous seed extract of *Moringa oleifera* (Moringaceae) were evaluated. Phytochemicals such as tannins, carbohydrates, alkaloids, cardiac glycosides, anthraquinones and flavonoids in low, moderate and high concentrations were present in the seeds. The extract exhibited significant *In vitro* antibacterial and *In vivo* haematologic effects. Bacterial isolates such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Corynebacterium pyogenes*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeroginosa* were inhibited. The minimum inhibitory concentration of the extract for all the sensitive isolates is 100 mg/ml and 50 mg/ml as minimum bactericidal concentration of the extract on *Staphylococcus aureus, Salmonella typhi* and *Escherichia coli*. The extract administered *per os* at 100-400 mg / kg body weight significantly ($P<0.05$) increased, neutrophil, eosinophil, basophil and monocytes counts at second and third week of administration except for lymphocyte that significantly ($P<0.05$) decreased throughout the period of administration when compared to the rats in control group. The study supports the folkloric application of *Moringa oleifera* seed extract in the management of various forms of bacterial infection, anaemia and immuno – modulation in north eastern Nigeria.

Keywords: Antibacterial effect, Haematology, *Moringa olifera*, Phytochemical analysis, Wistar albino rats.
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Introduction

Traditional Medicine is defined by World Health Organization as the sum total of knowledge or practices whether explicable or inexplicable used in diagnosing, preventing or eliminating a physical, mental or social disease which may rely exclusively on past experience or observations handed down from generation to generation, verbally or in writing (WHO, 1987).

Moringa oleifera (Moringaceae) is a popular plant in north eastern, Nigeria. The plant is used locally for various medicinal purposes by traditionalists and herbalists in Maiduguri, north eastern Nigeria. It has been reported to have an impressive range of medicinal uses with high nutritional value. *Moringa oleifera* have been traditionally used for its coagulative properties on waste water, cardiac and circulatory stimulant, antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial, antifungal activities and have lots of macro and microelements and various forms of nutrients (USDA, 2003; Katayon *et al*., 2005; Kebreab *et al*., 2005; Farooq *et al*., 2007).

The leaves particularly are used in the preparation of various delicacies among the Marghi, Kanuri,

Hausa/Fulani communities and many other tribes in the region. Various parts of the plant such as leaves, flowers, seeds, bark and roots is used in folklore medicine to manage ailments such as hypercholesterolemia, cellular oxidation, hyperglycaemia, hepatitis, bacterial and fungal diseases, ulcer, urolithiasis and cancer conditions (Fahey *et al*., 2002; Bennett *et al*., 2003; Bharali *et al*., 2003; Haristoy *et al*., 2005).

The objective of this study is to evaluate the phytochemical, antibacterial and haematologic properties of aqueous seed extract of *Moringa oleifera* (Moringaceae) in wister strain albino rats.

Materials and methods

Test for cardiac glycosides

Two millilitres of the aqueous solution of the extract was added 3 drops of strong solution of lead acetate. This was mixed thoroughly and filtered. The filtrate was shaken with 5ml of chloroform in a separating funnel. The chloroform layer was evaporated to dryness in a small evaporating dish. The residue was dissolved in a glacial acetic acid containing a trace of ferric chloride; this was transferred to the surface of 2ml concentrated sulphuric acid in a test tube. The upper layer and interface of the two layers were observed for bluish-green and reddish-brown colouration respectively, which indicates the presence of cardiac glycosides.

Test for steroids (Liebermann-Burchard's test)

The amount of 0.5g of the extract was dissolved in 10ml anhydrous chloroform and filtered. The solution was divided into two equal portions for the following tests. The first portion of the solution above was mixed with 1ml of acetic anhydride followed by the addition of 1ml of concentrated sulphuric acid down the side of the test tube to form a layer underneath. The test tube was observed for green colouration indicative of steroids.

Test for steroids (Salkowski's test)

The second portion of solution above was mixed with concentrated sulphuric acid carefully so that the acid formed a lower layer and the interface was observed for a reddish-brown colour indicative of steroid ring.

Test for flavonoids (Shibita's reaction test)

One gram (1g) of the water extract was dissolved in methanol (50%, 1-2ml) by heating, then metal magnesium and 5-6 drops of concentrated hydrochloric acid were added. The solution when red is indicative of flavonols and orange for flavones.

Test for flavonoids (pew's test)

To five millilitres (5ml) of the aqueous solution of the water extract was added to 0.1g of metallic zinc and 8ml of concentrated sulphuric acid. The reaction mixture was observed for red colour indicative of flavonols.

Test for anthraquinones (Borntrager's reaction for free anthraquinones)

One gram (1g) of the powdered seed was placed in a dry test tube and 20ml of chloroform was added. This was heated in steam bath for five minutes. The extract was filtered while hot and allowed to cool. To the filtrate was added equal volume of 10% ammonia solution. This was shaken and the upper aqueous layer was observed for bright pink colouration, which is an indication of the presence of anthraquinones. Control test were done by adding 10ml of 10% ammonia solution in 5ml chloroform in a test tube.

Antibacterial screening

Culture Media: Nutrient agar (Tab – Lemco powder 15.0g/l, Peptone, 10.0g/l, Sodium chloride, 5.0g/l, Agar, 15g/l) and Nutrient broth Tab – Lemco powder 15.0g/l, Peptone, 10.0 g/l, Sodium chloride, 5.0 g/l (Oxoid, England) of pH 7.3 were used for the investigation.

Microbial cultures: Laboratory isolates of *Streptococcus pyogenes, Staphylococcus aureus, Bacillus subtilis, Corynebacterium pyogenes, Klebsiella pneumoniae, Salmonella typhi, Enterobacter aeroginosa, Proteus mirabilis, Pseudomonas aeroginosa and Escherichia coli* were obtained from the department of Veterinary Medicine laboratory, University of Maiduguri, Nigeria. The isolates were cultured separately on nutrient agar plate for 24h. Twenty five (25ml) millilitre of the culture media was poured into sterile Petri dish and allowed to solidify. A colony of each test organism was sub cultured on 10 ml nutrient broth and incubated at 37^{o}C for 8 h. One millilitre of the sub-cultured organisms was inoculated on the agar plates.

Preparation of inocula: The inoculum size of all bacterial isolates tested was standardized by the use of overnight broth cultures prepared by inoculating isolated colonies of test bacteria in 10ml of Nutrient broth which was incubated at 35°C for 24 hours. A loopful of overnight broth culture was diluted in 4ml of sterile physiological saline (0.8% W/V), such that its turbidity marched with that of 0.5 Mac Farland standard (Barium sulphate standard) considered to have a mean bacterial density of 3.3×10^6 CFU/ml. This was gauged by comparing the turbidity of the test suspension with the turbidity 1% (W/V) Barium sulphate solution against the background of a printed white paper (Cheesbrough, 2002).

Preparation of antimicrobial discs: Graded concentrations of 200, 400, 600, 800 and 1000 mg/ml of the extract were measured and poured into different plates. About 1ml of sterile distilled water was added to each plate containing the extract and stirred. Filter paper discs (6mm) diameter were then placed in each plate and stirred so as to ensure the impregnation of the disc by the extract. Tetracycline (250 mg/ml) as the control drug was prepared and placed at the centre of each inoculated plate.

Incubation of bacterial isolates: The inoculated plates containing filter paper discs (6 mm) impregnated with the extract and control drug were incubated at 37°C for 18–24 hrs.

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration of the crude seed extract of *Moringa oleifera* was determined using the method of Greenwood (1989) as described by Geidam *et al.* (2007). Serial dilution of the extract at the concentrations of 200, 100, 50, 25 and 12.5 mg/ml respectively were used to determine minimum inhibitory concentration and recorded as the least concentration of the extract that completely inhibited the growth of the organisms.

Minimum bactericidal concentration (MBC)

Samples were taken from test tubes used in performing MIC assay and sub-cultured unto freshly prepared nutrient agar medium and later incubated at 37°C for 24hrs. The MBC was taken as the lowest concentration of the extract that inhibits bacterial growth on the agar plates (Olorundare *et al.,* 1992).

Haematological analysis

Blood sample collection: Blood samples were collected from the tail vein of the rats by snipping part of the tail for the determination of differential leucocytic count.

Determination of red blood cells (RBC) count: The method described by Coles (1986) was used for the red blood cells count determination using the improved Neubauer method. The erythrocyte diluting pipette was used to draw blood from the tail vein to exactly 0.5 mark. The tip of the pipette was wiped free of blood before inserting into the erythrocyte diluting fluid and the fluid drawn into the pipette up to the 101 mark above the bulb. The pipette was gently rotated and allowed to stand for 2 minutes. The first few drops from the pipette were discarded before being used to charge the counting chamber. The ruled areas of the haematocytometer were thoroughly and carefully cleaned to remove grease. The cover slip was then placed on the counting chamber which was thereafter charged with the fluid from the pipette. The chamber was left for 2 minutes, and cells in 5 of 25 small squares were counted under ×40 objective of light microscope. The number of the red cells counted were multiplied by ten thousand (10,000) to give number of the red blood cells in million per cubic millimeter (or $\times 10^6 mm^3$) (Coles, 1986).

Determination of the packed cell volume (PCV): Blood from the tail vein of the rat was allowed to run into the heparinzed capillary tube by capillary action until the tube is about three-quarter full. The end of the tube in contact with the blood was sealed with plastacine and placed in a micro- haematocrit centrifuged operated at the rate of 3,000 revolutions per minute (rpm) for 5 minutes, thereafter, the capillary tube was placed in a micro-haematocrit reader and the PCV read and expressed as percentage (Coles, 1986).

Determination of differential leucocytic count (DLC): A dry micropipette was used to suck blood from the snipped part of the rats tail, a small drop of blood was applied to one end of a slide and quickly placed on the bench holding it in position, the end of the second slide was then placed in the drop and held there until the blood had spread across it. Blood is dried and stained with giemsa, washed with distilled water and allowed to dry for 2 minutes and then examined with microscope at low and high power

magnification for cellular appearance (Osim *et al*., 2004).

Statistical analysis

The results are presented as Mean ± Standard deviation. Differences between means were assessed using Analysis of variance (ANOVA) and post test using Dunnett multiple comparison test (Mead & Curnow, 1982).

Results

The phytochemical evaluation of the aqueous seed extract of *Moringa oleifera* showed that tannins and carbohydrate occurred in low concentrations, saponins, alkaloids, cardiac glycosides, anthraquinones were present in the extract in moderate concentrations, while flavonoids is in high concentration, whereas phlobatannins and steroids were absent in the extract (Table 1).

With regards to the antibacterial activity, minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the extract is shown on tables 2, 3 and 4. Various bacterial isolates were inhibited with *Salmonella typhi* and *Klebsiella pneumoniae* being the most sensitive while *Staphylococcus aureus* and *Escherichia coli* were least sensitive to the extract where *Proteus mirabilis* and *Enterobacter aeroginosa* proved resistant. The minimum inhibitory concentration of the extract for all the sensitive isolates was 100 mg/ml whereas 50 mg/ml is the minimium bactericidal concentration of the extract that inhibited the growth of *Staphylococcus aureus, Salmonella typhi* and *Escherichia coli*.

The effect of the extract on haematology of Wister albino rats is clearly shown on Tables 5 and 6. There was significant ($P<0.05$) increase in packed cell volume (PCV), Red blood cell count (RBC) and white blood cell count (WBC), throughout 21 days of administration of 100, 200, 300 and 400 mg/kg when compared to the rats in control groups (Table 5). There was significant ($P<0.05$) increase in neutrophils, eosinophils, basophils and moncytes throughout the period of extract administration, except lymphocytic count that decreased throughout the period of administration (Table 6).

Table 1: Phytochemistry of the crude aqueous seed extract of *Moringa oleifera*

Phytochemical constituents	Test	Inference
Tannins	Ferric chloride	+
	Formaldehyde	+
	Chlorogenic acid	+
Saponins	Frothing	++
Alkaloid	Dragendorff's	++
	Mayer's	++
	Wagner's	++
Carbohydrates	Molisch's	+
	Barfoed's	+
	Combine reducing sugar	+
	Free reducing sugar	+
	Ketone's	+
	Pentose's	+
Phlobatannins	Hydrochloric acid	-
Cardiac glycosides	General test	++
Flavonoids	Pew's test	+
	Shibita's	+
Anthraquinones	Free Anthraquinones	++

\+ Low concentration ++ Moderate concentration +++ High concentration - Absent

Table 2: Antibacterial activity of crude seed extract of *Moringa oleifera* on some bacteria organisms

Extract /Antibiotic	Extract amount & Antibiotics (mg)	Zone of inhibition diameter(mm) Organisms									
crude extract of *Moringa oleifera*		*Staph aureus*	Strept. Pyogenes	*Bacillus subtilis*	Coryn. Pyogenes	*Kleb. Pneumoniae*	Salm typhi	*E. coli*	Pseud. Aeroginosa	Proteus mirabilis	Enterob. aeroginosa
	1000	11	18	10	8	17	18	10	8	R	R
	800	10	16	9	10	15	16	9	10	R	R
	600	9	12	8	12	13	14	8	12	R	R
	400	8	10	9	13	11	13	9	13	R	R
	200	7	9	9	16	10	10	9	16	R	R
Tetracycline	250	20	48	36	27	38	16	28	26	28	25

Key: R Resistant

Table 3: Determination of minimum inhibitory concentration (MIC) of *Moringa oleifera* crude seed extract

Organisms	Concentration of *Moringa oleifera crude* seed extract (mg/ml)				
	200	100	50	25	12.5
S. aureus	-	-	-	+	+
S. pyogenes	-	-	-	-	+
B. subtilis	-	-	+	+	+
C. pyogenes	-	-	-	+	+
K. pneumoniae	-	-	-	-	+
S. typhi	-	-	-	-	+
E. coli	-	-	-	-	+
P. aeroginosa	-	-	+	-	+

Key: + growth observed
- growth inhibited

Table 4: Determination of minimum bactericidal concentration (MBC) of *Moringa oleifera* crude seed extract

Organisms	Concentration of *Moringa oleifera crude* seed extract (mg/ml)				
	200	100	50	25	12.5
S. aureus	-	-	-	+	+
S. pyogenes	-	-	+	+	+
B. subtilis	-	+	+	+	+
C. pyogenes	-	-	+	+	+
K. pneumonia	-	-	+	+	+
S. typhi	-	-	-	+	+
E. coli	-	-	-	+	+
P. aeroginosa	-	-	+	+	+

Key: + growth observed
- growth inhibited

Table 5: The effect of the aqueous seed extract of *Moringa oleifera* on packed cell volume (PCV), red blood cell (RBC) and white blood cell (WBC) count in wistar strain albino rats

Dosage (mg/kg)	Period of Administration		
	1st week	2nd week	3rd week
	Packed cell volume (PCV)		
Control	45.4 ± 0.55	46.0 ± 0.71	45.8 ± 0.45
100	51.0 ± 0.71[b]	48.2 ± 0.84[b]	49.8 ± 0.45[b]
200	52.2 ± 0.84[b]	49.4 ± 0.55[b]	51.4 ± 0.55[b]
300	53.4 ± 0.55[b]	49.4 ± 0.89[b]	49.6 ± 2.30[b]
400	54.2 ± 0.84[b]	49.0 ± 1.41[b]	52.2 ± 1.30[b]
	Red blood cell (RBC) count		
Control	5.63 ± 0.27	5.49 ± 0.27	5.53 ± 0.26
100	7.29 ± 0.31[b]	6.65 ± 0.38[b]	6.97 ± 0.36[b]
200	9.22 ± 0.37[b]	7.88 ± 0.46[b]	8.16 ± 0.56[b]
300	8.62 ± 0.48[b]	7.63 ± 0.78[b]	7.45 ± 0.78[b]
400	9.85 ± 0.22[b]	8.66 ± 0.97[b]	9.14 ± 0.84[b]
	White blood cell (WBC) count		
Control	8506 ± 215.8	8490.0 ± 201.3	8493.8 ± 202.3
100	10640 ± 384.7[b]	8880.8 ± 257.9[a]	9462.0 ± 355.9[b]
200	12821.2 ± 336.0[b]	8785.4 ± 126.5[a]	10000.0 ± 291.6[b]
300	13080.2 ± 580.6[b]	9924.4 ± 331.2[b]	10320.8 ± 656.5[b]
400	14283 ± 191.6[b]	12120 ± 1308.4[b]	13582.0 ± 491.6[b]

a = Insignificant ($P > 0.05$) increase as compared to control
b = significant ($P<0.05$) increase as compared to control

Table 6: Effect of the aqueous seed extract of *Moringa oleifera* on differential leucocytic count (DLC) in wistar strain albino rats

Dose (mg/kg)	Treatment		
	1st week	2nd week	3 rd week
	Lymphocyte count		
Control	72.4 ± 2.07	72.0 ± 1.58	72.4 ± 2.07
100	67.6 ± 1.67[a]	60.6 ± 1.14 [a]	59.0 ± 1.58 [a]
200	67.0 ± 1.58 [a]	60.2 ± 2.86 [a]	59.9 ± 1.30 [a]
300	68.0 ± 1.58 [a]	62.2 ± 2.28 [a]	58.6 ± 2.51 [a]
400	68.8 ± 1.30 [a]	59.2 ± 1.64 [a]	58.4 ± 1.51 [a]
	Neutrophil count		
Control	21.6 ± 1.67	21.6 ± 1.14	21.6 ± 1.67
100	23.8 ± 1.92[a]	30.2 ± 1.48 [b]	31.4 ± 1.94 [b]
200	24.4 ± 2.07 [a]	30.6 ± 3.20 [b]	30.8 ± 1.48 [b]
300	22.4 ± 1.67 [a]	28.2 ± 2.77 [b]	30.6 ± 2.60 [b]
400	21.6 ± 1.14 [a]	30.6 ± 2.51 [b]	30.6 ± 1.94 [b]
	Eosinophil count		
Control	4.4 ± 0.54	4.6 ± 0.54	4.4 ± 0.54
100	5.6 ± 0.54 [b]	6.2 ± 0.83 [b]	4.6 ± 0.54[b]
200	5.7 ± 0.54 [b]	5.8 ± 0.44 [b]	6.0 ± 0.00 [b]
300	5.8 ± 0.83 [b]	6.2 ± 0.83 [b]	7.0 ± 1.22 [b]
400	5.4 ± 0.54 [a]	6.6 ± 0.54 [b]	7.0 ± 0.70 [b]
	Basophil count		
Control	0.6 ± 0.54	0.8 ± 0.44	0.6 ± 0.54
100	1.6 ± 0.54 [b]	1.4 ± 0.54 [b]	1.6 ± 0.89 [b]
200	1.8 ± 0.44 [b]	2.0 ± 0.70 [b]	2.0 ± 0.70 [b]
300	1.8 ± 0.44 [b]	1.6 ± 0.54 [b]	2.0 ± 0.70 [b]
400	1.0 ± 0.00 [b]	1.8 ± 0.44 [b]	2.0 ± 0.00 [b]
	Monocyte count		
Control	0.6 ± 0.54	0.8 ± 0.44	0.6 ± 0.54
100	1.6 ± 0.54 [b]	1.4 ± 0.54 [b]	1.6 ± 0.89 [b]
200	1.8 ± 0.44 [b]	2.0 ± 0.70 [b]	2.0 ± 0.70 [b]
300	1.8 ± 0.44 [b]	1.6 ± 0.54 [b]	2.0 ± 0.70 [b]
400	1.0 ± 0.00 [b]	1.8 ± 0.44 [b]	3.0 ± 0.00 [b]

a = significant (P<0.05) decrease as compared to control

b = significant (P<0.05) increase as compared to control

Discussion

The acute toxicity study conducted using the up and down method for the aqueous seeds extract is determined to be above 3000 mg/kg (Dixon, 1991; Dixon & Mood, 1984). Plant products with LD_{50} ranging between 50 - 500 mg/kg body weight are regarded as highly toxic, 500 - 1000 mg/kg moderately toxic, and above 1000 mg/kg are regarded as being of low toxicity (relatively safe) (Clark & Clark 1987) .

The aqueous seed extract of *Moringa oleifera* revealed high level of phytochemicals such as saponins, alkaloids, cardiac glycosides, flavonoids and anthraquinones as was reported by Josephine *et al.* (2010). The fractionated leaves portion reported by Arun & Purnachandra (2011) revealed low phytochemical contents when compared to that in *Moringa oleifera* aqueous seed extract. The *Moringa oleifera* aqueous seed extract in this study revealed the presence of free anthraquinones and cardiac glycosides which is in contrast with the finding of other researchers who worked on various parts *of Moringa oleifera*.

The aqueous seed extract of *Moringa oleifera* used in this experiment was found to be inhibitory on

many pathogenic bacteria, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacilus subtilis*, *Corynebacterium pyogenes*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeroginosa*. Fozia *et al.* (2012) reported similar activity, though in their research, no activity was reported on *Streptococcus pyogenes*, *Corynebacterium pyogenes*, *Klebsiella pneumoniae* and *Salmonella typhi.*

The antibacterial activity exhibited on some bacterial isolates by aqueous *Moringa oleifera* seed extract could be as a result of the presence of flavonoids and tannins, since these phytochemicals is reported to confer antibacterial activity (Cowan, 1999; Hausteen, 2005).

There was significant ($P<0.05$) increase in Packed cell volume (PCV), red blood cell count (RBC), white blood cell count (WBC), neutrophils, eosinophils, basophils and monocyte count, except lymphocytes throughout 21 days of administration of 100 - 400 mg/kg of the extract when compared to the rats in control groups, this effect could be due to the presence of high amount of flavonoids in the seeds of *Moringa oleifera* though, Ajibade *et al.* (2012) reported significant decrease in white blood cells count, neutrophils and monocytes count and insignificant changes in PCV, Hb, MCV, MCH and MCHC on using 800 and 1600 mg/kg methanol seed extract administered for 21 days. These variations could be due to the difference in geographical location, nature of the product used, dosage and concentration of phytochemicals in the aqueous and fractionated portions of *Moringa oleifera* seed used. Irene *et al.* (1998) and Cho *et al.* (2003) stated earlier that better therapeutic effect was recorded with aqueous products due to synergistic effect of the phytochemicals in them than in fractionated plant products.

The significant increase in the above parameters may be as a result of stimulatory effect of aqueous seed extract of *Moringa oleifera* on leucocyte. Flavonoids in the seeds of *Moringa oleifera* may be the reason for the leucocytosis observed, since flavonoids was reported to increase intracellular vitamin C synthesis, leucocytosis, decrease capillary permeability, fragility and have antioxidants property (Lee *et al*., 2003). Phytochemical components such as flavoniods in aqueous seed extract of *Moringa oleifera* is also shown to exert protective effect on chemically-induced hemolysis in G6PD deficient human and animal red blood cells, anti-inflammatory and antipyretic activity (Braide & Vitrotio, 1989; Braide, 1990).

The suppressive effect of the aqueous seed extract of this plant on lymphocyte counts may be due to the effect of some of the phytochemicals such as alkaloid and tannin in the seed. Reports have shown that components such as alkaloids, saponins, tannins and steroids may suppress the growth and differentiation of some cells in the bone marrow (Muller & Tobin, 1980).

Conclusively, the seed extract of *Moringa oleifera* has broadspectrum antibacterial activity and increases significantly red blood cells, Packed cell volume and white blood cells count based on the result of the differential leucocytic count obtained, though the seed had no effect on lymphocyte production.

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