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Evaluation of Antioxidant Activity of Ethanol Extract of Root and Stem Bark of *Moringa oleifera* (MO) obtained from Utu Ikpe, Ikot Ekpene Local Government Area, Nigeria

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Abstract: The work studied free radical scavenging activities of ethanol extract of the stem bark and root bark of MO. They are used by the local population to manage common health problems. Antioxidant activities of the plant parts extracts were studied spectrophotometrically using DPPH (1,1-dimethyl-2-picrylhydrazyl radical), (FRAP) ferric reducing antioxidant power, (H2O2) hydrogen peroxide, ABTS (2,2'azinobis (3-ethylbenzthiozoline-6-sulphonic acid), (NO) nitric oxide as basic free radicals. The result of phytochemical screening and quantitative content of the plant parts revealed a yield of (2.84%) and 4.34% for the stem and root barks respectively. Alkaloids (2.47%), saponin (0.169%), tannins (3.35%), flavonoids (1.34%) and Alkaloids (1.01%), saponin (0.03%) tannins (1.87%), flavonoids (traces) were estimated in the stem bark and root bark respectively. The total phenolic contents in the stem and root barks were (25.21 GAE/g) and (13.37 RE/g) of the dry samples respectively. The stem and root barks of the samples showed 5.30 AAE/g and 4.90 AAE/g respectively. Models of extracts on the basic free radicals were significant ($p<0.05$; $R2>90\%$) and acted linearly as a function of the phytochemical content and the free radicals' models in use. The IC50 of the stem bark and root bark on FRAP was 21.41±0.01 and 59.20±0.11 AAE/g respectively compared with ascorbic acid (40.56). The result indicated that ethanol extract of the plant parts may constitute a good source of natural compounds with antioxidant capability in the food and general health sector.

Keyword: *Moringa oleifera,* phytochemicals, free radical scavenging activity, free radical assay and reducing power

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1.0 Introduction

Plants play an important role in the health and nutrition of man (Abalaka *et al*., 2012; Fahey, 2005). They form a storehouse of phytochemicals and phytonutrients (Fahey, 2005). Most of the phytochemicals are known antioxidants when they are inactive synergy with phytonutrients, they can exhibit extraordinary functions in the prevention, delay and management of several health challenges in man (Sigh *et al.* 2011; Abalaka *et al.,*2012), and the nourishment of the mammalian system (Fahey., 2005). For example, there exists a strong and positive correlation between consumption of total flavonoids, total phenolic content, carotenes and good health. Embaby and Mokhtar, (2011) reported that *Lantana camara* and *Cucubita pepo* demonstrated good antioxidants *in vitro*. MO is indigenous to the Indian sub-continent, but now it grows in most West African countries (El-Sohaimy *et al.,* 2015). The plant thrives at all agronomic conditions, even when and where other crops cannot, therefore it is available and renewable all year round (Fahey, 2005). The stem bark and the root bark of the plant are significant in the traditional medical practice of the local folks. They perceive the plant parts to be anti-astringent, anti-cool, cardiotonic, expectorant, and anti-ulcers. The root bark extract is commonly used in the treatment of cough, otalgia, tumor, bronchitis, asthma, fractures (Garima *et al*., 2013; Rebecca *et al*., 2006; Singh *et al.,* 2012).

The present work aimed to investigate the radical scavenging activity of ethanol extract of stem bark and root bark of MO with the specific objectives of i. to screen the extract for phytochemicals, ii. determine total phenolic content (TPC) and total flavonoid contents (TFC), iii. estimate the polyphenol contents and iv. determine the percentage oxidation inhibition and reduce the power of the ethanol extract using some stable free radicals; 1, 1-dimethyl-2-picrylhydrazyl (DPPH), 2,2'azinobis (3-ethylbenzthiozoline-6-sulphonic acid (ABTS), Ferric reducing antioxidant power (FRAP). Hydrogen peroxide (H_2O_2), and nitric oxide (NO). The result of the work is expected to ascertain the claim of antioxidant activity of ethanol extract of the plant parts.

2.0 Material and methods

DPPH (1,1-dimethyl-2-picrylhydrazyl radical), (FRAP) ferric reducing antioxidant power, ABTS (2,2'azinobis (3-ethylbenzthiozoline-6-sulphonic acid), (H_2O_2) hydrogen peroxide, (NO) nitric oxide, ethanol, aluminum nitrite, sodium hydrogen phosphate, and potassium persulphate, sodium nitrite, rutin, gallic acid, sodium hydroxide were of analyte grade obtained from Pauli Chemical Shops, Ikot Ekpene, Nigeria.

2.1 Collection of plant materials

The stem bark and root bark of MO were harvested from Utu Ikpe Village in Ikot Ekpene Local Government Area, Nigeria. The plant had been earlier identified at the Botanical Unit of the School of Applied Sciences, Akwa Ibom State Polytechnic, Ikot Ekpene. Nigeria where the specimen samples are stored. The samples were cleaned of extraneous materials, washed with distilled water and air-dried to constant weight at room temperature (28±2.0oC). The clean sample was chopped into tiny bits, shade-dried at room temperature for 20 days and grounded to a powder and stored for use.

2.2 Preparation of ethanol extracts of MO

Ethanol extract of the samples each was obtained using the method reported by Embaby and Mokhtar, (2011). One (1) kg of the powder sample was macerated in 2 L of 95% ethanol, shaken vigorously and allowed to stand for 84 h. The mixture was filtered through No. 1 Whatman filter paper, the filtrate was collected in a 250 ml conical flask and evaporated to dryness under reduced pressure in a rotary evaporator (Rotor, Buchi, Switzerland). The percentage crude extract yield was calculated using equation 1.

$$\text{Yield} = \frac{\text{Weight of extract}}{\text{Weight of sample}} \times 100 \quad (1)$$

2.3 Preliminary phytochemical screening of the samples

The preliminary phytochemical screening of the samples was carried out according to the method reported by Saforowa, 2008; Thilagavathi, 2015) to test for the presence of alkaloids, flavonoids, saponins, tannins, in the samples.

2.3.1 Quantitative estimation of phytochemicals

Quantitative estimation of tannins, alkaloids, flavonoids and were determined according to the methods described by Ejikeme, *et al*. (2014), Chukuwuma and Chigozie (2016), Ejikeme, *et al.* (2014), Amadi *et al*. (2004) respectively.

2.3.2 Estimation of total phenolic content

The total phenolic content (TPC) of the sample was estimated according to the method adopted by Silverton and Rossi (1965). One (1) mL of the extract was introduced into a 25 mL volumetric flask and mixed with 10 mL of distilled water and 1.5 mL of Folin-Ceocalteu's reagent. After 5 minutes, 4 mL of 20% (w/v) sodium carbonate solution was added, the volume mixture was made up to 25 mL with distilled water. After 30 min, the absorbance of the reaction mixture was read at 765 nm. The total concentration of phenolic compounds was estimated by extrapolation from the calibration curve of gallic acid.

2.3.3 Determination of total flavonoid contents

The total flavonoid content of the samples was evaluated using aluminum chloride $AlCl_3$) according to the method adopted by Embaby (2011) in the presence of rutin as standard. 0.1 mL of the extract was added to 0.3 mL of distilled water, followed by the addition of 0.03 ml of 5% $NaNO_2$ solution. The reaction mixture was incubated for 5 min at room temperature (28.0±1.0 ºC). Another 0.03 mL 10% of $AlCl_3$ was added and kept for 5 min, before treatment with 0.2 mL (mM) NaOH. The reaction mixture was diluted to a 1 ml mark with distilled water and the absorbance was measured at 510 nm using a UV/Vis spectrophotometer (Virian Australia Pty. Ltd., Victoria, Australia). The run was done in triplicate and the concentration was evaluated through the calibration curve.

2.4 Estimation of antioxidant activities

2.4.1 Free radical scavenging activity of crude extract of MO on DPPH

The antioxidant activity of the sample was carried out according to the method adopted by Polthum and Ahromrit (2014). A conical flask was filled with 50 µL of each extract, at a concentration of 1 to 5 mg/mL 5 mL of 0.004% (w/v) solution of DPPH was added to the reaction mixture. The mixture was vortexed, incubated for 30 min in the dark at room temperature (28±1.0ºC). The absorbance of the mixture was measured with a UV/Vis spectrophotometer (Virian Australia Pty. Ltd., Victoria, Australia) at a wavelength of 517 nm. The ascorbic acid standard curve was prepared using a serial solution of 1 to 5 mg/mL. The control did not contain any antioxidants, values of absorbance of the control were subtracted from that of the control. The capacity of the plant extract to scavenge the DPPH radical was estimated using equation 2.

$$\text{Percentage inhibition} = \frac{Ao - As}{Ao} \times 100 \quad (2)$$

where A_o = absorbance of the mixture without a sample (blank), A_s = absorbance of mixture with samples. Lower absorbance values of the reaction mixture indicated higher scavenging activity.

2.4.2 Ferric reducing antioxidant power (FRAP) assay

The determination of the free radical scavenging capacity of the extracts was carried out using the method of Benzie and Stain (1999). The FRAP reagent was obtained according to the method of Benzie and Strain, (1996) as adopted by Ujah *et al*. (2021). The volume of 300 mM sodium acetate buffer (pH 3.6), 10 mM (tripyridyl triazine) TPTZ solution and 20.0 mM 100 10.0 mM (tripyridyltriazine) TPTZ solution and 20.0 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 v/v/v. Concentrations range (25, 50, 75, 150 and 300 µg/mL) of the extract were added separately to 3.0 mL of the FRAP reagent. The reaction mixture was incubated at 37ºC for 30 min for complete reaction and formation of colour. The absorbance of the reaction mixture was measured at 593 nm. A fresh working solution of ascorbic acid was used to construct the calibration curve. The antioxidant capacity of the sample extract was based on its ability to reduce ferric ions. The sample was calculated from the linear calibration curve of TPTZ and expressed as mmol $FeSO_4$ equivalent per gram of sample dry weight. Higher absorbance indicated greater reducing power of the sample extract.

2.4.3 Hydrogen peroxide scavenging activity

The free radical scavenging capacity of the extracts on Hydrogen peroxide was carried out according to the method of Kumar *et* al. (2012). Hydrogen peroxide solution (2 mM) was prepared with standard phosphate buffer (pH 7.4). Different concentrations of the extracts of (20 - 100 µg/mL) was added to 1.0 mL of Hydrogen peroxide solution. The absorbance of the reaction mixture was determined at 320 nm after 10 min. The percent oxidation inhibition of the extract was calculated according to equation 3. Each run was carried out in triplicate.

$$\text{Percentage inhibition} = \frac{Ao-As}{Ao} \times 100 \qquad (3)$$

where, A_0 = the absorbance of hydrogen peroxide radical and ethanol, A_s = the absorbance of Nitric oxide radical and sample extract/standard.

2.4.4 Nitric oxide scavenging activity

Nitric oxide free radical scavenging assay was conducted using the method adopted by Vikash *et al.,* (2012). 3 mL of 10 mM sodium nitroprusside in phosphate buffer was respectively added to 2.0 mL of the extract of the reference compound in different concentrations of 20-100 µg/mL to produce nitrite ions. The resulting solutions were incubated at 25 °C for 60 minutes. Similar treatment was repeated with ethanol as control. Five (5.0) mL of Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylene diamide dihydrochloride in 2% H_3PO_3) was added to the incubated sample, the absorbance of the coloured reaction mixture formed was measured at 540 nm. Percent inhibition of the nitric oxide generated was measured by comparing the absorbance of the ascorbic acid standard.

$$\text{Percentage inhibition} = \frac{\text{Ao}-\text{As}}{\text{Ao}} \times 100 \qquad (6)$$

where A_o and A_s are the absorbances of the control and that of the sample

2.5 ABTS scavenging activity

The ABTS free radical scavenging activity of the sample extract was determined according to the method adopted by Ujah *et al.* (2021). The cation, $ABTS^{+\cdot}$ was generated by persulfate oxidation of ABTS. A mixture of 2:1, v/v of ABTS (7.0 mM) and Potassium persulfate (4.95 mM) was incubated in the dark at 30±2.0 °C for 24 h to form radical cations of ABTS. A working solution was diluted with ethanol to obtain absorbance values of 0.70±0.02 µg at 734 nm. Fifty (50) µl aliquot of each sample was mixed with 3 ml of the working solution,

the decrease in absorbance was measured at 734 nm after incubation in the dark for 10 min. The percentage inhibition of the free radical by the plant extract was calculated using equation

$$ABTS^{+} \text{ scavenging } (\%) = \frac{Ao - A}{Ao} \times 100 \quad (7)$$

where A_o = absorbance of control solution, A_s = absorbance of sample and ABTS ion.

2.5.1 Determination of reducing the power of the extracts of parts of MO

The reducing power of ethanol extract of MO parts was determined according to the methods of Benzie and Stain. (1996) and Vikash *et al.* (2012). The concentration of 20-100 µg/mL of ethanol extracts were mixed with phosphate buffer (5.0 mL, 2.0 M, pH 6.6) and 1% potassium ferrocyanide (4 mL), and the reaction mixtures were incubated at 50 ^{o}C for 20 min. Five (5) mL of 10% Trichloroacetic acid was added, the mixture was centrifuged at 550 x g for 10 min. 5 mL of the upper layer of the solution was taken and mixed with 5 mL of distilled water followed by 0.1 % Ferric chloride (1 mL). The absorbance of the mixture was measured using a UV/visible spectrophotometer (Virian Australia Pty. Ltd., Victoria, Australia). At 700 nm. The reducing power of the plant extracts on the free radicals was determined using the linear curve of the standard. The experiment was conducted in triplicate and results were averaged and expressed as mean ± SD.

3.0 Results and Discussion

Table 1 presents results obtained from the preliminary phytochemical screening which included detected ions tested for alkaloids, flavonoids, saponin and tannin. The concentration of the identified phytochemicals is also shown in the same Table 1.

Table 1: Qualitative and quantitative analytical results for the phytochemicals

Phytochemical	EESB		EERB	
	Qualitative	Quantitative	Qualitative	Quantitative
Alkaloids	+	1.84^{a}±0.12	+++	1.00^{b}±.01
Flavonoids	++	1.34^{a}±0.02	-	-
Saponin	+	0.169±.01	+	0.03±.03
Tannin	++	3.35^{a}±0.31	++	1.87^{b}±0.02

Values are means^{n}±SD of triplicate determinations. Values in the same column not bearing the same superscript are significantly different (p≤0.05).. Key: +++ = high presence, ++ = moderate presence, + low presence, – not detected. A=undetected, AL=alkaloid (%), SA=saponin (%), TA=tannin (mg/100g), FL=flavonoid (%),

From the information recorded in Table 1, the concentration of alkaloids was low in EESB but very high in EERB. Flavonoid was not detected in the EERB but moderate concentration was found in the EESB. However, both the root and stem barks indicated the presence of saponin at low concentration while moderate contents of tannin were recorded for both the stem and root barks. The concentration of alkaloids 1.84 in ESSB and 1.0 µg/g in ESSB µg/g respectively. Flavonoids in the extract were 1.34 and 0.01 µg/g, saponin showed 0.16 and 0.03 µg/g, while tannin showed 3.35 and 1.87 µg/g in EESB and EERB respectively. The distribution of phytochemicals in the plant parts is obvious and may arise from differences in biosynthesis processes and retention of the compounds in the plant parts. The variation could also be attributed to the season and agronomical environment of the plant. Kumari, (2016) opined that the aerial parts of plants are more favourable to the production of phytochemicals than the stem and the lower parts of plants (Ogbe and Afiku 2011; Ujah *et al.,* 2021). The presence of alkaloids, flavonoids, and saponin in the plant stems and root barks provides strong backing for their reported use for pharmaceutical or medicinal reasons. Several

literatures have supported the potential antibiotic activities of some phytochemicals on the mammalian systems, especially in the prevention against infection (Bathra and Sharma, 2013; Fahey, 2005; Bathra and Sharma, 2013; Tundis et al., 2013; Yen and Che 1995) Flavonoids possess antioxidants activity, hence extracts of the part of a plant that is rich in phytochemical molecules may be used to treat free radical related health problems in man (Meryem *et al*. 2016). Flavonoids can also exhibit anti-inflammatory, anti-carcinogenic and nephroprotective bioactivity. Tannin has been commended for its role in the healing of stubborn wounds because it is a polyphenol with a high molecular weight that facilitates the formation of a protective coverage on wounds (Okwuomu *et al*., 2017). The presence of alkaloids may be supportive of the traditional applications of plant parts for the management of malaria, body pain and stomach disorder (Anonymous, 2021). Alkaloids have also been found to exhibit several biological activities such as anti-malaria, anti-hyperglycemia, anti-cancer, anti-cancer, in addition to the stimulating roles of some members of the group (for example; sanguinarine, morphine, and codeine) (Okwuonu *et al*., 2017).

Table 3: TPC and TFC of SBE and RBE of *MO*

Samples	Percentage Yield	TPC (µg (GAE/mg)	TFC (µg RE/mg)
Stem bark	2.84^{b}±0.03	^{a}25.21±0.14	^{a}5.30±0.03
Root bark	4.34^{a}±0.01	^{b}13.37±0.40	^{a}4.90±0.09

Values not preceded by the same superscript are different, at 0.05 confidence level, TPC = total phenol content, TFC = total flavonoid content

The crude extract yield, total phenolic content, and the total flavonoids content of EESB and EERB of MO are presented in Table 3. The table shows the crude extract from the stem bark yielded 2.84% while that of the root bark yielded 4.34%. The TPC from stem bark (25.21 GAE/mg) was higher than that of the root bark (13.37 GAE/mg). Also, the TFC from the stem bark (5.30 µg RE/mg) showed a slight difference from that of the root bark (4.90 µg RE/mg). Sangeeta and Vrunda (2016) and Thanh *et al.* (2017) reported a 5.55% of the parameter in the root of MO obtained from Pakistan, which is in strong agreement with the present data. Thanh *et al.* (2017) and Iqbal and Bhanger (2006) attributed the variations in the yield of crude extract of the stem and root bark to the maturity of the plant at harvest, the season of harvest, and environmental factors of growth. The results indicate that the stem bark contained more TPC than the root bark the observation was in agreement with the trend of differences in crude extracts and phytochemicals reported by Kumari *et al.* (2016) on the distribution of phytochemicals and bioactivity in different parts and leaf positions *Stevia rebaudiana* and Singh and Shama (2012) on 'antimicrobial evaluation of leaf extract of *MO* lam.

Table 4: Free radical scavenging activity of EESB and EERB of *MO*

	DPPH	FRAP	H_2O_2	NO	ABTS	AA
EESB	48.10±.12	70.40±.01	69.50±.04	42.90±.02	69.50±.02	72.10
EERB	51.20±.03	69.23±.02	71.20±.04	42.50±.03	71.20±.01	72.10

**** TPC = total phenolic content of the extract, TFC = total flavonoid content of the extract, EESB = ethanol extract of stem bark of *MO,* EERB =ethanol extract of root bark of *MO***

The free radical scavenging activity of EESB and EERB of MO on the stable free radicals is shown in Table 3. The model of the EESB on the basic free radical assays was significant ($p<0.05$) and reliable, $R^2 > 90\%$. The free radical scavenging activity of the highest concentration of the extracts ranged from 48.10 to 59,50%, the highest value was observed in FRAP followed by H_2O_2, 69.50 %, and ABTS, 60.50 % compared with 72.10% of AA. The lowest value was observed in DPPH, 48.10%. Model of the EERB was significant ($p<0.05$, $R^2 > 90\%$). The highest concentration of the plant extract revealed the free radical scavenging activity of the extract ranged between 42.50 to 71.20% respectively. The effect of the extract on H_2O_2 and ABTS shows the highest value of the parameter of 71.20 and 72.21 %. The values of the parameter were not in agreement with previous works, the variations could be attributed to post-harvest handling, nature of the samples, agronomical factors under which the plant was grown (Garima *et al.,* 2011).

The reducing power of EESB and EERB of ethanol extract of MO on the stable free radicals is presented in Table 6. According to the table, the free radical scavenging activity of the stem bark extract on DPPH, FRAP, H_2O_2, NO, and ABTS ranged from 50.72 to 65.15 AAE/g dw of the sample. The highest value was observed in the plant extract on FRAP, 21.41, H_2O_2 (50.72) AAE/g dw of the sample. The value was comparable with that of ascorbic acid (40.56). The observation shows that the plant extract could be a source of herbal, powerful antioxidants. The root bark extract followed the pattern of the stem bark and was equally comparable with the standard. The high reducing values on DPPH, NO and ABTS may depend on the mechanism of the electron transfer process during the reaction or the amount and concentration of the phytochemicals in the plant parts (Gordon, 1992). The observation is similar to the values reported by (Ujah *et al.,* 2021). The slight differences between the IC_{50} values could be attributed to the agronomical environment of the plant parts and the experimental design in use.

Table 6: Reducing power, IC_{50} (AAE/g) of ethanol extracts of stem bark and root bark of MO

Sample	DPPH	FRAP	H_2O_2	NO	ABTS	AA
Stem bark	65.15±.12	21.41±.01	50.72±.21	68.09±.23	63.07±.12	40.56±0.01
Root bark	65.01±.01	59.20±.02	62.03±.13	71.62±.11	62.87±.09	40.56±0.01

****Values are means±SD of triplicate determinations. Values in the same column not, bearing the same superscript are significantly different ($p\leq0.05$), DPPH = 2,2-diphenyl-1-picrylhydrazylH_2O_2 = Hydrogen peroxide, ABTS = 2, 2' azinobis (3-ethylbenzothiazoline-6-sulphonic acid, AA = Ascorbic acid, NO = Nitric oxideIC_{50} = Mean lethal concentration, *In-vitro* antioxidant activity**

4.0 Conclusion

The study found that the stem bark and root bark contain phytochemicals, exhibit a high level of free radical scavenging activity, and good reducing power all comparable with the ascorbic acid standard. Therefore, it is highly likely that there is a high probability that the plant extracts can be used in the treatment of some free radical related health problems in man. The work was limited by time and finance; therefore, it is continued to include histopathological analysis of animals treated at an extended period before full introduction into the health system.

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Competing interests

The authors declared no competing interest.