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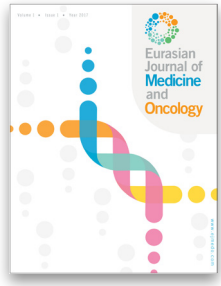

Research Article

In vitro and *in vivo* Determination of Hydroxyl Radical Scavenging Activity (HRSA) of Fractions of Aqueous Extract of *Moringa Oleifera* Leaves (AEMOL)

Ahmad Adekilekun Tijani,[1] Damilare Adedayo Adekomi,[2] Stephen Oderinde Adewole[3]

[1]Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, Ekiti State University, Ado-Ekiti, Nigeria
[2]Department of Anatomy, Faculty of Basic Medical Sciences, College of Health Sciences, Osun State University, Osogbo, Nigeria
[3]Department of Anatomy and Cell Biology, Faculty of Basic Medical Sciences, College of Health Sciences, Obafemi, Awolowo University, Ile-Ife, Nigeria

Abstract

Objective: Humans are exposed to endogenous and exogenous sources of free radicals almost daily. Free radicals are harmful as they can impact several metabolic pathways in the cells. Oxidative species known for such harmful effects are reactive oxygen species, reactive nitrogen species, hydroxyl radical, and hydrogen peroxide. The presence of these free radicals in humans has been documented to lead to many pathological conditions, including lethality, mutagenesis, carcinogenesis, aging, and also degenerative diseases. *Moringa oleifera* Lam is the best known and most widely distributed species of the Moringaceae family, with an impressive range of medicinal uses with high nutritional value worldwide. The leaves of this plant are traditionally known for and reported to have various biological activities, including free radical scavenging effect. The aim of the present study was to determine the fraction of aqueous extract of *Moringa oleifera* leaves (AEMOL) with the most outstanding scavenging activity.

Methods: In our study, the *in vitro* and *in vivo* hydroxyl radical scavenging activities of fractions (chloroform, ethyl acetate, and N-hexane) of AEMOL were investigated.

Results: *In vivo* examination of ethyl acetate, chloroform, and N-hexane fractions of AEMOL showed that ethyl acetate exhibited outstanding scavenging effect that might be due to its ability to provide a particularly effective way of maximizing the bioavailability of the active phytochemical substances extracted from the plant, which is also in conformity with our *in vitro* findings.

Conclusion: The mode of action appears to be that ethyl acetate acts to keep the active components in the solution after ingestion, thus facilitating their absorption into the bloodstream.

Keywords: Aqueous extract, Hydroxyl radical scavenging activity, *in vitro*, *in vivo*, *Moringa oleifera* leaves

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Humans are exposed to endogenous and exogenous sources of free radicals almost daily. These sources could be exposure to UV light, cigarette smoke, ionising radiation, certain organic solvents, pollutants and industrial waste.[1] Lead as a major environmental pollutant is one of the four metals (cadmium, mercury and arsenic) that have the most damaging effects on human health.[2] Free radicals are harmful as they can impact several metabolic pathways in cells. Oxidative species known for such harmful effects are the reactive oxygen species, reactive nitrogen species, hydroxyl radical and hydrogen peroxide.[3] The presence of these free radicals in human beings might lead to the aetiology of many pathological conditions including lethality, mutagenesis, carcinogenesis, aging and also degenerative

Address for correspondence: Ahmad Adekilekun Tijani, MBBS; Ph. D. Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, Ekiti State University, Ado-Ekiti, Nigeria
Phone: +923480380635 82 **E-mail:** tijani.adekilekun@eksu.edu.ng
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diseases such as coronary heart disease and Alzheimer's disease.[4] Although these free radicals can be scavenged by the *in vivo* produced antioxidant compounds, the endogenous antioxidants are insufficient to completely remove them and maintain a balance. Dietary antioxidants are therefore required to counteract excess free radicals.[5-7]

Consumption of antioxidants in food or as supplements can help protect the body against these diseases.[8] Because antioxidants act as a major defence against radical mediated toxicity by protecting the damages caused by free radicals. They mainly function as free radical scavengers, chain breaking antioxidants, metal chelators, reducing agents, oxidative enzyme inhibitors and quenchers of singlet oxygen.[9] Recently special attention has been paid towards edible plants, especially those that are rich in phytochemicals. The phytochemicals are capable of combating the free radicals efficiently. The phenolics are major chemical identity among the phytochemicals. This group of compounds is principally correlated with their antioxidant property.[10-12] Phenolic compounds are widely distributed in fruits and vegetables. Polyphenols can donate hydrogen to free radicals and generate relatively non reactive reduced forms, thus, acting as chain breaking antioxidants.[13]

Natural antioxidants are recommended over their synthetic counterparts (butylated hydroxyanisole and butylated hydroxytoluene) because they are viewed as less toxic and more potent than synthetic antioxidants.[1] Natural antioxidants such as vitamin C, tocopherols, flavonoids and other phenolic compounds are known to be present in certain plants.[14] *Moringa oleifera* Lam is the best known and most widely distributed species of Moringaceae family, having an impressive range of medicinal uses with high nutritional value throughout the world. Leaves of this plant are traditionally known for or reported to have various biological activities, including hypocholesterolemic agent, regulation of thyroid hormone status, anti-diabetic agent, treatment of gastric ulcers, anti-tumor agent and hypotensive agent.

In this study, the *in vitro* and *in vivo* hydroxyl radical scavenging activity of fractions (chloroform, ethyl acetate, N-hexane) of aqueous extract of *Moringa oleifera* leave (AEMOL) were investigated.

Methods

Sample Collection and Authentication

Fresh mature leaves of *Moringa oleifera* were obtained from Obafemi Awolowo University campus, Ile-Ife between the hours of 9.00 and 11.00. The plant was authenticated by a taxonomist at the Department of Botany, Obafemi Awolowo University, Ile-Ife and a voucher specimen deposited at the herbarium of the department with reference number IFE 17379.

Sample Preparation and Extraction

The leaves were washed and air dried at room temperature in the Drug Research Unit of the Faculty of Pharmacy of the University. 100g of the dried leaves were pulverized in a warring blender to yield 85g of Moringa powder. 100 g of the pulverized leaves was extracted with distilled water at 80^{0}C and 30 extraction cycles for 3 hours, using Soxhlet extractor and according to the Edward Randall's modified Soxhlet Method, described by Anderson (2004) to obtain a dark green extract of 23.5g. Aqueous extract obtained was successively re-extracted using N-hexane, chloroform and ethyl acetate in order of increasing polarity and the fractions were tested separately *in vitro* and *in vivo* to determine their hydroxyl radical scavenging activities.

In vitro Determination

Aliquots (3 mg/ml) of the fractions of *Moringa oleifera* leave extract (N-hexane-, chloroform- ethyl acetate- and aqueous extracts) were prepared and tested *in vitro* for their Hydroxyl Radical Scavenging Activity (HRSA).

Assay of Hydroxyl Radical Scavenging Activity (HRSA)

The hydroxyl radical scavenging activity (HRSA) was measured by studying the competition between deoxyribose and the fractions for hydroxyl radicals generated from the Fe^{3+}/ascorbate /EDTA/H_2O_2 system according to the method of Barry et al.[15]

Procedure: The reaction mixture contained 1.0 ml of reagent (3.0 mM deoxyribose, 0.1 mM EDTA, 2 mM H_2O_2, 0.1 mM L-Ascorbic acid, 0.1 mM $FeCl_3.6H_2O$ in 10 mM phosphate buffer, pH 7.4) and various concentrations of the extracts (50-350 µg/ml). The reaction mixtures were incubated at 37 oC for 1 h, and followed by the addition of 1.0 ml of 1% (w/v) TBA (in 0.25 N HCl) and 1.0 ml 10 % (w/v) TCA. The reaction mixtures were heated in boiling water bath at 100 oC for 20 minutes and the pink chromogen (malondialdehyde-(TBA) adduct) was extracted into 1.0 ml of butan-1-ol for the absorbance to be read at 532 nm against reagent blank. The percentage inhibition was calculated using the expression:

$$\text{Percentage inhibition} = \frac{\text{Abs (control)} - \text{Abs (sample)} \times 100}{\text{Abs (control)}}$$

In vivo Determination

Animal Procurement, care and Maintenance

Thirty (30) adult male Wistar rats weighing between 130g and 150g were procured from the animal holding of the College of Health Sciences, Obafemi Awolowo University, Ile-Ife. The rats were housed in plastic cages in the animal holding of the Department of Anatomy and Cell Biology. They were

maintained on standard laboratory rat pellets and given water *ad libitum*. Ethical clearance for the research was obtained from Health Research Ethics Committee (HREC) of the Institute of Public Health (IPH), Obafemi Awolowo University, Ile-Ife. The animals received humane care according to the guidelines of HREC.

Experimental Design and Administration

The rats were randomly divided into six (6) groups A-F with each group containing 5 rats kept in the same cage. Group A was the positive control group and rats in the group received 100 ml/kg of distilled water for 15 days. Group B was the negative control group and rats in the group received 10 mg/kg of lead nitrate solution for 15 days. Groups C, D, E and F were the test groups and rats in the groups received 10 mg/kg of lead nitrate solution for 15 days, followed by 400 mg/kg of hexane-, chloroform-, ethyl acetate- and aqueous-fractions of AEMOL respectively for another 15 days. All administrations were oral.

Animal Sacrifice and Collection of Blood/Serum Sample

Each animal was sacrificed by chloroform ether anaesthesia 24 hours after the last administration. Blood sample was taken from the apex of the heart into a 5 ml plain serum bottle. Plain samples bottles containing blood samples from the animals were made to slant in the sample bottles' rack to allow blood clotting. This was followed by spinning for 5 minutes at 4000 revolutions per minutes to get the serum which was stored at – 20°c. Serum were assayed for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GSH) and malondialdehyde (MDA) for *in vivo* measurement of antioxidant potency of the fractions of AEMOL.

Statistical Analysis

The data obtained was analysed using the computerized statistical package SPSS version 21. One-way ANOVA was used to compare the mean and standard error of mean values within and between groups of experimental animals, followed by Tukey HSD test for multiple comparisons and

Table 1. *In vitro* Mean Inhibition Concentrations [IC 50 (mg/ml)] of fractions of AEMOL for Hydroxyl Radical Scavenging Activity (HRSA)

Extracts	Concentrations (mg/ml)	% Inhibition		Mean % Inhibition
		A1	A2	
Standard (trolox)	500.00	93.65	99.34	96.50
	250.00	78.89	78.69	78.79
	125.00	56.76	57.99	57.38
	62.50	34.63	38.73	36.68
	31.25	22.54	18.24	20.39
	IC 50 (mg/ml)=0.14			
Aqueous Extract (Control)	1.50	85.25	84.22	84.73
	0.75	75.61	75.20	75.41
	0.375	73.36	71.72	72.54
	0.1875	43.03	41.60	42.32
	0.09375	19.47	15.78	17.62
	IC 50 (mg/ml)=0.33			
Ethyl Acetate Fraction	1.50	72.95	78.48	75.72
	0.75	60.04	63.93	61.99
	0.375	52.25	46.52	49.39
	0.1875	24.80	33.81	29.30
	0.09375	15.57	12.09	13.83
	IC 50 (mg/ml)=0.68			
Chloroform Fraction	1.50	93.96	77.67	75.82
	0.75	55.12	52.46	53.80
	0.375	34.63	31.76	33.20
	0.1875	24.80	21.31	23.05
	0.09375	1.22	2.46	1.84
	IC 50 (mg/ml)=0.84			
N-Hexane Fraction	1.50	60.25	55.94	58.09
	0.75	24.80	32.58	28.69
	0.375	11.48	11.48	11.48
	0.1875	1.84	1.03	1.43
	0.09375	-0.82	-0.21	-0.51
	IC 50 (mg/ml)=1.29			

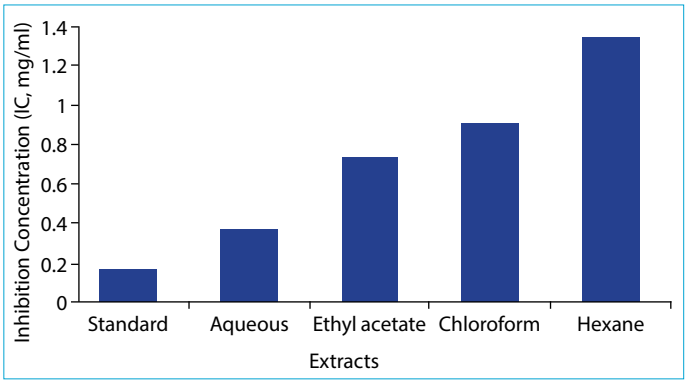

Figure 1. Bar chart of the *In vitro* Mean Inhibition Concentrations [IC 50 (mg/ml)] of fractions of AEMOL for Hydroxyl Radical Scavenging Activity (HRSA).

determination of the significant difference between these mean values. Probability values less than 0.05 were considered statistically significant.

Results

In vitro Determination

Hydroxyl Radical Scavenging Activity (HRSA) of each fraction of AEMOL was determined as mean percentage inhibition and was compared with one another and with those of a standard solution and AEMOL (as control) (Table 1). 0.14mg/ml of the standard solution scavenged 50% of the hydroxyl radicals generated while 0.33mg/ml, 0.68mg/ml, 0.84mg/ml and 1.25mg/ml of AEMOL, ethyl acetate, chloroform and N-hexane fractions respectively scavenged the same amount (50%) of the generated hydroxyl radicals. The lowest (Fig. 1) concentration of ethyl acetate fraction (0.68mg/ml) scavenging/inhibiting 50% of generated free radicals made it to be most potent of the fraction of AEMOL for HRSA.

In vivo Determination

Markers of oxidative stress (superoxide dismutase–SOD, catalase–CAT and glutathione peroxidise–GPX) and lipid peroxidation (Malondialdehyde–MDA) were determined in the serum of animals treated with AEMOL and its fractions following lead (Pb) exposure. Values were given in table 2 as mean±SEM for five (5) animals in each group. Multiple group comparison following one way ANOVA analysis using Tukey HSD post hoc test (Table 3) indicated a statistically significant difference between the mean value of the group of animals that was not exposed to lead nor treated with AEMOL or its fractions (A) and the group (B) that was treated with lead alone for all parameters (SOD, CAT, GPX and MDA). There were also statistically significant differences between the mean values of the group (A) that was not treated at all and that of the group (C) treated with N-hexane fraction after lead exposure for SOD and GPX and between the mean values of group A and that of the group (D) treated with chloroform fraction after lead exposure for SOD. There were no statistically significant difference between the mean values of group A and groups E and F treated respectively with Ethyl acetate fraction and AEMOL after lead exposure for all parameters of oxidative stress

Table 2. Descriptive analysis of *in vivo* concentrations of markers of oxidative stress and lipid peroxidation

Group	N	SOD Mean±SEM	CAT Mean±SEM	GPX Mean±SEM	MDA Mean±SEM
A	5	103.58±0.20	6.37±0.25	150.01±1.01	3.13±0.05
B	5	95.47±0.20	4.74±0.19	132.46±0.82	3.87±0.04
C	5	98.57±0.66	5.75±0.14	145.37±1.03	3.09±0.08
D	5	100.67±0.61	5.94±0.12	146.77±1.09	3.06±0.08
E	5	02.70±0.19	5.97±0.10	149.94±0.56	2.99±0.09
F	5	103.25±0.28	6.75±0.17	152.43±0.69	3.07±0.07

Table 3. Multiple comparison (Tukey HSD) of *in vivo* concentrations of markers of oxidative stress and lipid peroxidation

(I) Group	(J) Group	SOD Mean±SEM/Sig	CAT Mean±SEM/Sig	GPX Mean±SEM/Sig	MDA Mean±SEM/Sig
A	B	*8.12±0.58/0.000	*1.63±0.24/0.000	*17.55±1.25/0.000	*-0.73±0.10/0.000
	C	*5.02±0.58/0.000	0.62±0.24/0.140	*4.64±1.25/0.013	0.04±0.10/0.999
	D	*2.92±0.58/0.000	0.43±0.24/0.486	3.25±1.25/0.139	0.07±0.10/0.980
	E	0.90±0.58/0.633	0.40±0.24/0.557	0.07±1.25/1.000	0.15±0.10/0.701
	F	0.340±0.58/0.991	-0.38±0.24/0.624	-2.42±1.25/0.411	0.62±0.10/0.990

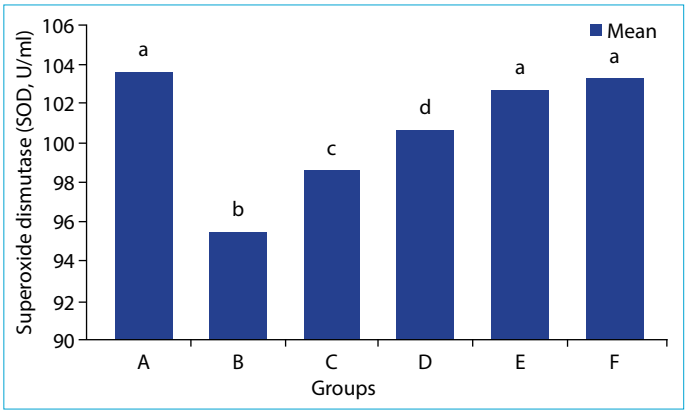

Figure 2. Bar chart of Mean Serum Superoxide Dismutase of Groups of Animals administered with fractions of AEMOL for Hydroxyl Radical Scavenging Activity (HRSA).

NB: bars with different alphabets have means that are statistically significantly different from one another.

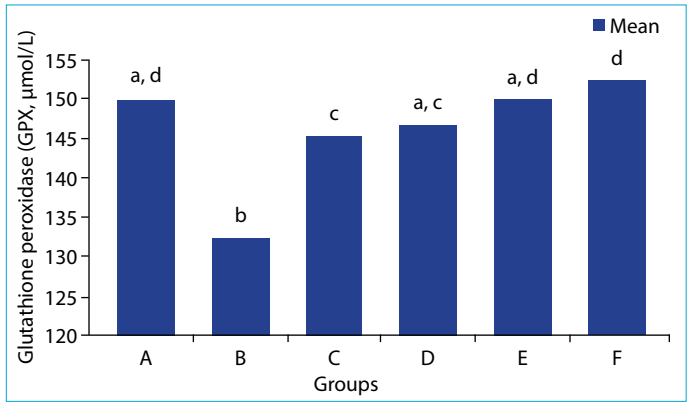

Figure 4. Bar chart of Mean Serum Glutathione Peroxidase of Groups of Animals administered with fractions of AEMOL for Hydroxyl Radical Scavenging Activity (HRSA).

NB: bars with different alphabets have means that are statistically significantly different from one another.

and lipid peroxidation (Figs. 2–5). Table 4 (a – d) showed the results of homogenous categorisation of the various mean values for the groups of animals. Categorisation was done for alpha of 0.05 and the homogenous subsets for each parameter of oxidative stress and lipid peroxidation were displayed in the tables. Homogenous subsets are group of animals with no significant difference in their mean values.

Discussion

In Vitro free Radical Scavenging Activity

The therapeutic value of medicinal plants lies in the various chemicals present in it. In the earlier study of phytochemical analysis carried out on *Moringa oleifera* leaves by Ojiako,[16] tannins, saponins, phenols, alkaloids, and phlobatannins are the major secondary metabolites found in appreciable quantity. The data on hydroxyl radical scavenging activity are given in Table 1. It clearly showed that all the extracts have the ability to scavenge generated hydroxyl radical but at different concentration. Apart from water which is a non polar solvent, polarity of other extracts varies. Followed by water are ethyl acetate, chloroform and hexane in that order. The results as depicted in Figure 1. showed that the lowest fraction of ethyl acetate scavenged 50% of generated hydroxyl radicals. This might be due to its polarity closer to that of water. Also, ethyl acetate extract appears to be more effective because polar extracts are known to show more phytochemicals than the non polar extract. Chloroform and hexane are sometimes categorised as non polar solvent. This indicates that the activity of the extract is influenced by the solvent used for the extraction and is in

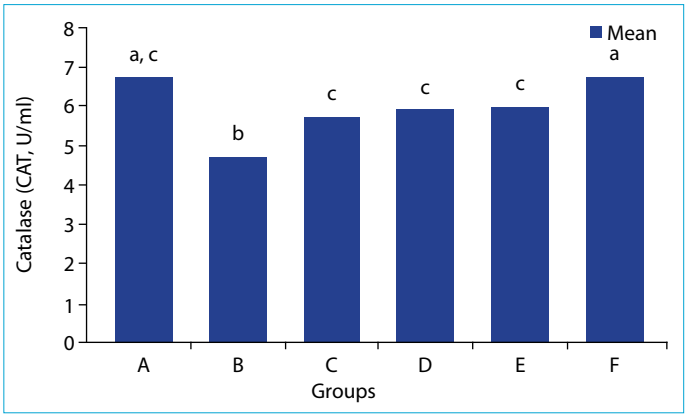

Figure 3. Bar chart of Mean Serum Catalase of Groups of Animals administered with fractions of AEMOL for Hydroxyl Radical Scavenging Activity (HRSA).

NB: bars with different alphabets have means that are statistically significantly different from one another.

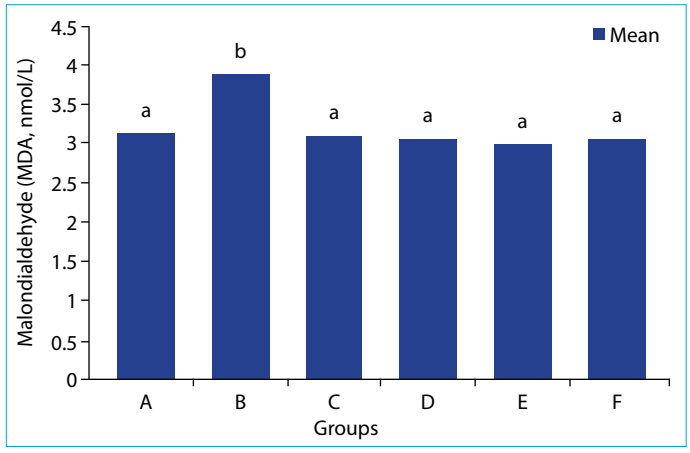

Figure 5. Bar chart of Mean Serum Malondialdehyde of Groups of Animals administered with fractions of AEMOL for Hydroxyl Radical Scavenging Activity (HRSA).

NB: bars with different alphabets have means that are statistically significantly different from one another.

Table 4. Homogenous Subset for comparison of *in vivo* concentrations of markers of oxidative stress and lipid peroxidation

(a) Superoxide Dismutase (SOD)					
Tukey HSD					
Group	**N**	**Subset for alpha=0.05**			
		1	2	3	4
B	5	95.4740			
C	5		98.5680		
D	5			100.6740	
E	5				102.6960
F	5				103.2540
A	5				103.5920
Sig.		1.000	1.000	1.000	.633
(b) Catalase (CAT)					
Tukey HSD					
Group	**N**	**Subset for alpha=0.05**			
		1	2	3	
B	5	4.7400			
C	5		5.7540		
D	5		5.9420		
E	5		5.9700		
A	5		6.3720	6.3720	
F	5			6.7480	
Sig.		1.000	.140	.624	
(c) Glutathione Peroxidase (GPX)					
Tukey HSD					
Group	**N**	**Subset for alpha=0.05**			
		1	2	3	4
B	5	132.4600			
C	5		145.3740		
D	5		146.7660	146.7660	
E	5			149.9400	149.9400
A	5			150.0140	150.0140
F	5				152.4300
Sig.		1.000	.873	.139	.379
(d) Malondialdehyde (MDA)					
Tukey HSD					
Group	**N**	**Subset for alpha=0.05**			
		1	2		
E	5	2.9860			
D	5	3.0620			
F	5	3.0720			
C	5	3.0940			
A	5	3.1340			
B	5		3.8680		
Sig.		.701	1.000		

accordance with the finding of Ojiako,[16] who reported that MO leaf extract is indeed a very useful breakthrough in the demand of alternative natural medicine for the treatment of various disease activities by pathogenic organisms.

In Vivo Free Radical Scavenging Activity

In the *in vivo* study, oxidative stress mediated by lead exposure are exhibited by a significant decrease in the activities of antioxidant enzymes superoxide dismutase–SOD, cata-

lase–CAT and glutathione peroxidise–GPX and significant increase in the activities malondialdehyde –MDA. This is in contrast with the findings of Patra et al.,[17] who reported that changes in the superoxide dismutase and catalase activities in lead-exposed rats did not reach statistical significance ($p<0.05$).

SOD is a metal-containing antioxidant enzyme that reduces harmful free radicals of oxygen formed during normal metabolic cell processes to oxygen and hydrogen peroxide and it also participates in the body's defence system against oxidative stress.

Significant increase in Catalase activity in fractions of AEMOL treated group is in accordance with the findings of Omotoso et al.,[18] who reported administration of AEMOL with lead afford significant increase in catalase activitiy.

Pachauri et al.[19] not only demonstrated that low dose (0.1%) of lead stimulate biomarkers such as ROS and GSH indicative of oxidative stress with lead-induced toxic manifestations in blood, kidney and brain but also severe depletion of antioxidants like SOD, catalase and glutathione peroxidase along with specific lead biomarker, blood ALAD (Delta-aminolevulinate dehydratase) in lead intoxicated animals.

In the present investigation, *in vivo* examination of ethyl acetate, chloroform and N-hexane fractions of AEMOL shows that ethyl acetate exhibited outstanding scavenging effect that might be due to its ability to provide a particularly effective way of maximizing the bioavailability of the active phytochemical substances extracted from the plant. This is also in conformity with our *in vitro* findings above. The mode of action seems to be that ethyl acetate acts to keep the active components in solution after ingesting, thus facilitating their absorption into bloodstream.

Conclusion

As per our knowledge, different solvent extracts of AEMOL in the *in vitro* and *in vivo* examinations showed the hydroxyl radical scavenging action depending on the type of solvent. Further studies are needed to specify the phytochemical constituents that are better extracted by each of the solvent.

Disclosures

Ethics Committee Approval: The study was approved by the Local Ethics Committee.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – A.A.T.; Design – A.A.T.; Supervision – O.S.A.; Materials – A.A.T., D.A.A.; Data collection &/or processing – A.A.T., D.A.A.; Analysis and/or interpretation – A.A.T., D.A.A.; Literature search – D.A.A.; Writing – A.A.T.; Critical review – O.S.A.

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