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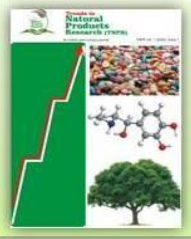

In vitro and *in vivo* antioxidant potential of crude extract and fractions of *Mallotus oppositifolius (Geisel.) Euphorbiaceae*

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Abstract: Excessive generation of oxygen free radicals plays a pivotal role in the destruction of biological molecules like DNA, proteins, lipids, carbohydrates and results in various pathologies including neuronal disorders. Antioxidant molecules from natural products are reported to have ability to mitigate their production or at least halt their progression and metastasis in the system. Different studies have been performed to spot antioxidants from natural sources and attempts have been made to integrate them in conventional therapy. In this study, methanol extract and fractions of *Mallotus oppositifolius* have been evaluated for in vitro and in vivo antioxidant potential. Different concentrations of the methanol extract and fractions were subjected to different in-vitro antioxidant assays (DPPH, ferrous ion chelating and nitric oxide scavenging activities). The in-vivo effects of the extract and fractions (250 mg/kg and 500 mg/kg), on the activities of serum and liver superoxide dismutase (SOD), catalase (CAT), Malondialdehyde (MDA), Glutathione peroxidase (GPX) and Glutathione (GSH) were measured in rats. Levels of oxidative stress biomarkers were measured. Results obtained depicted that *M. oppositifolius* root extract demonstrated a dose-dependent pattern in its efficacy. The extract was able to scavenging the DPPH, ferrous and nitric oxide, as well as returning the level of the oxidation biomarkers to normal rang. These results suggested that the *Mallotus oppositifolius root* extract and fractions exhibited a significant antioxidant activity both in-vitro and in-vivo that can be an important source of natural antioxidants.

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INTRODUCTION

Ironically Oxygen, an element vital for life, at some points becomes destructive for human body and the most deleterious effects result from the production of free radicals and reactive oxygen species (ROS), (Lobo *et al.,* 2010). Free radical is depicted as any chemical moiety, which has an unpaired electron in its orbital and thus has the tendency to donate/accept an electron from other molecules (Galano 2015). The most significant oxygen containing free radicals contributing towards many pathophysiological problems are hydroxyl, superoxide anion, hydrogen peroxide, nitric oxide, and peroxynitrite radicals (Birben *et al.,* 2012). These highly reactive and unstable species are capable of causing damages and the most vulnerable targets are biologically important molecules such as DNA, proteins, carbohydrates, and lipids. Apart from physical damages this leads to havoc in the homeostasis of body. Oxidative stress has been interconnected to numerous chronic diseases (Galano 2015). For instance, increasing evidence suggested a pathological impact of oxidative stress in the development of complications of the two major types of diabetes mellitus (Maritim *et al.,* 2003). The main reactive species include reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS are generated in human body due to external and internal physiological processes. However, the imbalanced production of the oxidants can lead to the damage of many biomolecules (Nimse and Pal 2015). Normally, animal cells are equipped with many defense mechanisms against oxidative stress including glutathione (GSH), vitamins C and E, catalase (CAT), superoxide dismutase (SOD) and various peroxidases (Krystone*t al.,* 2011). Basically, antioxidants counteract the oxidation of biological molecules by delaying or inhibiting mechanism (Willcox *et al.,* 2004). Early modulations of oxidative stress by exogenous natural antioxidants and diet rich in vitamins have proved beneficial in the protection against the oxidative stress induced damage (Rice-Evans *et al.,* 2016). Plant polyphenols; have gained considerable attention due to their possible health benefits. Epidemiological studies showed an effective impact of polyphenol plant diets on the reduction of the incidence of cancers, diabetes, osteoporosis, cardiovascular and neurodegenerative disorders (Pandey and Rizvi 2009).

Mallotus oppositifolius (Geisel.) Müll. (M. oppositifolius) belongs to the family Euphorbiaceae. I It is a shrub known in tropical Africa for its medicinal applications. The herb has been used therapeutically to cure a variety of illnesses. The stick is chewed for dental hygiene and teeth cleansing, while the root is decocted to treat anaemia, pneumonia, and as an aphrodisiac (Idu *et al.,* 2007). More specifically, the ethanolic and aqueous leaf extracts of *M. oppositifolius* have been reported to have antifungal, antibacterial, and antimalarial properties (Chukwujekwu *et al.,* 2005), antioxidant and antidiabetic activities (Onyeka et al*.,* 2021). It is reported to contain phytochemicals appreciable amount of minerals essential vitamins (Onyeka *et al.,* 2021). Also, previous study has reported that the root extract of the plant had anti-inflammatory, antioxidant, antidiarrheic, antibacterial, antifungal and antitrypanosomal properties (Kabran *et al.,* 2012). About 32 compounds was isolated from the leaf, stem and bark (Refs). The plant has also been reported for activity against pathogenic yeasts while three compounds including betulinic acid, quercetine and quercitin were isolated from methanol leaf extract (Ngouana *et al.,* 2021). The aim of this study was to evaluate the in vitro and in vivo antioxidant potential of the crude extract and fractions of *Mallotus oppositifolius* roots.

MATERIALS AND METHODS

Reagents

All reagents and chemicals were supplied and purchased from Sigma-Aldrich, USA unless otherwise specified.

Plant material

M. oppositifolius root was collected in Delta State. Plant material was identified by the plant taxonomist Dr. Theophilus Ebukafrom the Department of Botany Sciences, Nnamdi Azikiwe University, Nigeria.

Animals

Albino rats of either sex (120-155 g) were procured from the animal facility, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were housed in the Animal House, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu. They were given access to feed and water *ad libitum.* Animals were handled in compliance with the National Institute of Health Guidelines for the care and use of laboratory animals for research purposes (Pub No. 85-23).

Preparation of crude extract and fractions

The root of *M. oppositifolius* were chopped into small pieces grinded with a grinding machine. 600 g of the dried grinded root was cold-macerated using methanol 80% for crude extraction. 155 g of the crude extract was obtained. Fractionation of the crude extract was achieved by liquid-liquid partitioning of the extract with n-hexane, ethyl

acetate, and butanol as described by Omoirri *et al.* (2020). The crude extract (65 g) was dispersed in 250 ml of distilled water. The solution was poured into 1000 ml separating funnel and was mixed with n-hexane (500 ml) properly by inversion and allowed to stand until two phase separation was achieved. The upper layer (n-hexane partition) was separated and the lower portion was subjected to fresh n-hexane solvent until the upper layer was clear. After n-hexane phase, the remaining portion was extracted with ethyl acetate followed by butanol successively. The left over served as the water fraction. All the fractions obtained were filtered and concentrated to dryness using a water bath set at 40°C.

Phytochemical Analysis

The crude extract and fractions were screened for the presence of secondary metabolites following standard procedures described by Harborne (1973).

Total phenolic and flavonoid contents analysis

Folin-Ciocalteu assay was used to evaluate and analyze the total phenolic contents as described by Singleton *et al* (1999). The results were expressed as mg/g gallic acid equivalent. The colorimetric aluminium chloride assay was used to evaluate and determine the total flavonoid content expressed as mg/g quercetin.

Antioxidant activity in vitro

DPPH radical scavenging assay

The radical scavenging activity of *M. oppositifolius* root extract and fractions was determined by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, and Ascorbic acid (vitamin C) was used as a standard as described by Dontha (2016). Briefly, 1 mL of different concentration (5-500 µg/mL) of the root extract was added to 2 mL of 0.1 mM of DPPH/methanol solution, followed by 30 minutes of incubation in dark conditions. Finally, the optical density was recorded at 517 nm against scavenger-free blank.

Ferrous ion (Fe^{2+}) chelating assay

Ferrous ions chelating activity was conducted as described by Mathew *et al.,* (2006). Briefly, 3 mL of root extract at different concentrations and was added to 0.25 mL of 2 mM $FeCl_2$solutionand 0.2 mL of 5 mM ferrozine solution was added for reaction initiation and left at room temperature for 10 minutes. EDTA solution was used as a positive control. Finally, the optical density was measured at 562 nm against the blank.

Nitric oxide scavenging assay

This assay was carried out following the method described by Habibure *et a*l (2013). Two millilitre of 10 mM sodium nitroprusside prepared with phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of samples at various concentrations (6.25, 12.5, 25, 50, 100. 200, 400 and 800 pg/ml). The mixture was incubated at room temperature for 150 min. Thereafter, 0.5 ml of the reaction mixture was withdrawn and mixed with 0.5 ml of Griess reagent (1% sulphanilamide + 0.1% naphthyl ethylenediamine dichloride + 3% phosphoric acid) was added to each test tube. The absorbance of the pink chromophore was measured at 540 nm. Ascorbic acid and Quercetin were used as standards

In vivo Antioxidant Study

Acute toxicity test

Lorks (1983) method was employed in this study. Three doses (10, 100 and 1000 mg/kg) were administered in the first phase and the animals were observed for 24 hours. In the second phase, four dose ranges were used 2000, 3000, 4000 and 5000 mg/kg body weight and were also observed for another 24 hours for sign of toxicity and death.

Animal treatment

Albino wistar rats (130) were used for this study. Three days after induction of diabetes, with alloxan monohydrate, the animals were divided into 13 groups of 10 rats each. The fasting blood glucose level of the animals was monitor daily a Glucometer (Accu-Check, Roche). The animals were treated p.o for 28 days as follows:

Group 1: Control rats received normal saline (10ml/kg)
Group 2: Diabetic control received normal saline (10ml/kg)
Group 3: Standard Drug (Metformin (100mg/kg)
Group 4: Ethanol extract (250mg/kg)
Group 5: Ethanol extract (500mg/kg)
Group 6: n-hexane fraction (250mg/kg)
Group 7: n-hexane fraction (500mg/kg)
Group 8: Ethyl acetate fraction (250mg/kg)
Group 9: Ethyl acetate fraction (500mg/kg)
Group 10: Butanol fraction (250mg/kg)
Group 11: Butanol fraction (500mg/kg)
Group 12: Water fraction (250mg/kg)
Group 13: Water fraction (500mg/kg)

On 28th day, the animals were fasted for 12 h, weighed and anesthetized with ether, blood was collected through ocular puncture, the blood was centrifuge and the serum obtained was use for the antioxidant test.

In vivo antioxidant activity

Serum was isolated from blood samples by centrifugation at 3000 rpm for 6 min at 4°C. The liver was homogenized in phosphate buffer saline. After centrifugation at 15000 rpm for 15 min at 4°C, the supernatant was used for oxidative stress assessment. CAT, SOD MDA, GPX and GSH enzyme activity were measured using Amplite TM Fluorimetric CAT, SOD, MDA, GPX and GSH Assay Kit (AAT Bioquest, USA) according to the manufacturer's instructions.

Statistical analyses

The results were expressed as mean ± Standard deviation. Statistical analysis of data was performed by one way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons. P values ($P < 0.05$; $P < 0.01$; $P < 0.001$) were considered as significant. IC_{50} were determined using Graph pad prism software (5.0).

RESULTS

Phytochemical analysis

Phytochemical evaluation of the revealed the presence of steroids, tannins, saponins, alkaloids, flavonoids, terpenoids, phenols, anthraquinones, cardiac glycosides, reducing sugars, proteins and amino acids in the crude extract and fractions of *Mallotus oppositifolius*

Acute toxicity test

There were no signs of toxicity nor death recorded in the acute toxicity study even at the dose of 5000 mg/kg. Therefore, the LD50 was taken to be above 5000mg/kg.

***In-vitro* antioxidant**

Total flavonoid content, total phenolic content and DPPH scavenging activity

Total phenolic content was found to be highest in n-hexane fraction (85.43±0.41mgGAE/g) followed by ethyl acetate fraction (49.87±1.32mgGAE/g)**.** The total flavonoid content in the n-hexane fraction was also the highest (127.21±1.54mgQEq/g) (table 1).

Lowest IC_{50} value was obtained with the n-hexane fraction (9.43±0.88µg/ml) followed by ethyl acetate fraction (12.32±1.33µg/ml) while methanol crude extract had the highest IC_{50} (21.33±0.54µg/ml) in DPPH scavenging activity of extract and fractions (Table 1).

Ferric reducing antioxidant power (FRAP) and nitric oxide (NO)

The reducing power of the extract and fractions increased with increase in the concentrations. The n-hexane fraction exhibited the highest activity with EC_{50} (12.31±1.76µg/ ml) while water fraction had the lowest activity with EC_{50} (16.20±0.88 µg/ ml), Ascorbic acid had EC_{50} of (10.22±1.31µg/ ml).
In Nitric oxide scavenging assay, n-hexane fraction also exhibited a maximum scavenging activity with IC_{50} of (7.60±1.74µg/ml) The standards ascorbic acid and Quercetin had (7.32±0.16µg/ ml and 8.54±0.12µg/ ml) respectively (Table 2).

***In-vivo* antioxidant results**

There was a significant decrease ($P<0.05$) in SOD CAT, GPx, GSH and elevation in the level of MDA in STZ treated rats. Oral administration of the extracts, at the dose of 250 and 500 mg/kg elicited a significant ($P < 0.001$) increase in SOD, CAT, GPx, GSH and decrease in MDA. N-hexane gave best activity, when compared to other treatments (Table 3)

Table 1: Total flavonoid content, total phenolic content and DPPH scavenging activities of extract and fractions.

	Total phenolic content (mgGAE/g)	Total flavonoid content (mgQEq/g)	DPPH, scavenging activity, IC_{50} (µg/ml)
Methanol crude extract	25.76±0.54	44.21±0.32	21.33±0.54
n-hexane fraction	85.43±0.41	127.21±1.54	9.43±0.88
Ethyl-acetate fraction	49.87±1.32	55.87±0.77	12.32±1.33
Butanol fraction	21.32±0.12	32.65±0.84	15.12±0.54
Water fraction	24.76±0.59	38.32±2.31	29.61±0.67
Ascorbic acid	87.12±0.85	132.43±0.53	9.54±0.31
Quercetin	92.15±0.48	-	10.14±0.33

Values are presented as mean ± SEM

Table 2: Ferric reducing antioxidant power (FRAP), Nitric oxide (NO) scavenging activities of the extract and fractions

	FRAP, EC_{50} (µg/ml)	NO scavenging activity, IC_{50} (µg/ml)
Methanol crude extract	32.54±1.44	21.45±0.25
n-hexane fraction	12.31±1.76	7.60±1.74
Ethyl-acetate fraction	14.66±1.27	10.42±0.21
Butanol fraction	21.26±1.65	21.12±0.45
Water fraction	16.20±0.88	17.21±2.90
Ascorbic acid	10.22±1.31	7.32±0.16
Quercetin	11.09±1.68	8.54±0.12

Values are presented as mean ± SEM

Table 3: Effect of the extract and fractions on activities of SOD, CAT, MDA, GPX and GSH in STZ induced diabetic rats.

GROUP	TREATMENT	SOD (U/mg protien)	CAT (µg/mg)	MDA (µg/mg)	GPX (U/mg protein)	GSH (ug/mg protein)
1	Normal Control	8.65±0.07***	82.14±0.40**	48.14±1.21***	44.45±0.32***	1.68 ± 1.04***
2	DC (STZ 50mg/kg)	3.34±0.08#	38.54±0.44#	98.83±0.54#	24.33±0.42#	1.12 ± 0.49#
3	Metf 100mg/kg	8.24±0.05	84.43±0.60	45.52±0.32	45.65±0.76	1.67 ± 0.66***
4	CE 250mg/kg	6.85±0.07***	80.14±0.50***	49.14±1.21***	40.33±0.64***	1.55 ± 0.51***
5	CE 500mg/kg	6.11±0.07***	78.09±0.49***	54.89±0.77**	35.99±0.50**	1.48 ± 0.42**
6	NHF 250mg/kg	7.87±0.06***	83.55±0.43***	46.76±0.42***	42.26±0.53***	1.58 ± 0.27***
7	NHF 500mg/kg	8.60±0.09	82.09±0.21***	48.21±0.44***	44.12±0.32***	1.68 ± 1.33***
8	EAF 250mg/kg	6.40±0.07***	78.65±0.30***	54.45±1.11***	40. 10±0.31***	1.50 ± 0.71***
9	EAF 500mg/kg	6.66±0.08***	80.15±0.27***	49.20±1.44***	44.09±0.86**	1.68 ± 0.25***
10	BTF 250mg/kg	6.60±0.08***	78.63±0.27***	52.36±0.29**	40.10±0.86**	1.73 ± 0.19***
11	BTF 500mg/kg	7.95±0.08***	82.34±0.80***	48.34±0.28***	42.22±0.54***	1.45 ± 0.32***
12	WF 250mg/kg	6.17±0.09***	78.42±0.60***	52.15±0.65**	35.54±0.50**	1.40 ± 0.14**
13	WF 500mg/kg	7.03±0.05***	81.46±0.87***	50.56±0.60***	44.37±0.11***	1.77 ± 0.66***

All the values are expressed as mean ±SEM n/group =10. Data were analyzed by ONE WAY ANOVA followed by Dunnett's multiple comparisons test. #P<0.001: significant difference from normal control. **P<0.01; ***P<0.001. compared with respective diabetic control. Where: DC =Diabetic control, Metf= Metformin, CE= Methanol crude extract, NHF= N-hexane fraction, EAF= Ethyl acetate fraction, BTF= butanol fraction and WF= water fraction.

DISCUSSION

The phytochemical screening showed that *M. oppositifolius* root extract is rich in bioactive constituents indicating its medical benefits. Oxidative stress is generated when the free radicals and oxidants are produced in excess which can damage many biological molecules that are important for cell functions (Willcox *et al.,* 2014). The oxidative stress has been shown to have high impact in many disorders like neurodegenerative diseases, cancer and diabetes (Pandey and Rizvi 2009). Since scavenging of free radicals could inhibit the harmful effect of free radicals and stop the spreading of oxidation (Krystone*t al.,* 2014), the antioxidants contents of plant extract through their scavenging activity are valuable for management of those diseases (Pandey and Rizvi 2009). Scientific evidence suggests that the flavonoids and phenolic acids, the most studied groups of polyphenols, play an essential role in protecting cell constituents against oxidative damage (Pandey and Rizvi 2009). In the present study, the *M. oppositifolius* extract and fractions had very high total phenolic and flavonoids contents.

The *in vitro* antioxidant assay revealed a potent antioxidant activity comparable to vitamin C which was used as a reference standard. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a dark-coloured crystalline powder composed of stable free radical molecules. In laboratory, it is used to monitor chemical reactions involving radicals, most notably antioxidant assay (Sharma and Bhat 2009). The antioxidant compounds neutralize the free radical character of DPPH by transferring either electrons or hydrogen atoms to DPPH (Naik *et al.*, 2003), thereby changing the colour from purple to the yellow-coloured stable diamagnetic molecule diphenyl picrylhydrazine. The degree of discoloration indicates the scavenging potential of the extract or antioxidant in terms of hydrogen donating ability (Mosquera et al., 2007).

The FRAP assay is also regarded as an imperative marker of the antioxidant potential of any test sample. It bestowed a direct inference of the antioxidants or reductants available in the sample. In FRAP assay, reduction of ferric (Fe^{3+})/(Fe^{2+}) ferrous couple took place. In this assay, blue-colored ferrous tripyridyl triazine complex at acidic pH was monitored spectrophotometrically. The activity was concentration dependent, as the extract of *M. oppositifolius* displayed the highest antioxidant capacity at the highest concentration. The evidence of the free radical scavenging potential of the extract was further compared by investigating its ability to scavenge nitric oxide (NO) production. Despite the possible beneficial effects of NO, its contribution to oxidative damage is increasingly becoming evident. Our result showed that *M. oppositifolius* root extract had more potent nitric oxide scavenging activity than the standard ascorbic acid. The nitric oxide level in all the assay media increased with time as depicted by the increase in nitrite levels.

The *in-vivo* antioxidant assay showed that the extract increased the activity of serum SOD, CAT, GPX, GSH and decreased the serum level of TBARS. Catalase is a ubiquitous enzyme that catalyzes the decomposition of hydrogen peroxide, a reactive oxygen species, which is a toxic product of both normal aerobic metabolism and pathogenic ROS production (Kohen and Nyska 2002). The SOD catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen, thereby reducing the likelihood of superoxide anion reacting with nitric oxide to form reactive peroxynitrite (Maritim *et al.,* 2003). The increased serum activities of catalase and SOD as observed in this study suggest that the extract has an in-vivo antioxidant activity and is capable of ameliorating the effect of ROS in biological system (Manonmani *et al.,* 2005). Also, ROS react with all biological substance; however, the most susceptible ones are polyunsaturated fatty acids. Reactions with these cell membrane constituents lead to lipid peroxidation (LPO) (Bakirel *et al*., 2008). Increased LPO impairs membrane function by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptor (Arulselvan and Subramanian 2007). Thiobarbituric acid reactive substance (TBARS) levels were measured as a marker of LPO and malondialdehyde (MDA) production. Malondialdehyde is an endogenous genotoxic product of enzymatic and ROS-induced LPO whose adducts are known to exist in DNA isolated from healthy human being (Niedernhofer *et al.,* 2003). In our study, the level of TBARS in the extract treated groups decreased in a dose dependent manner when compared to control. This decrease in the TBARS levels may indicate increase in the activities of glutathione peroxidase and hence inactivation of LPO reactions (Niedernhofer *et al.,* 2003). It was shown that the level of GPX and GSH were reduced by *M. oppositifolius* root extracts. They have long been used as antioxidant enzymes to play an important role in combating oxidative stress and maintaining redox balance. Glutathione peroxidase (GPx) is a key reactive oxygen species (ROS) scavenger that combines with catalase (CAT) and superoxide dismutase (SOD) to form an antioxidant defense system (Do *et al.,* 2019). GPx utilizes a reducing agent to eliminate hydrogen peroxide and lipid peroxides, preventing peroxidative damage to the cell membrane and other organelles by regulating glutathione (GSH) (Tolomeo *et al.,* 2016). GPx is responsible for

recycling GSH as well as regulating GSH regeneration (Hansen *et al.,* 2006). GSH is an electron donor in the redox process of GPx (Salminen *et al.,* 2014). Some of the phytochemical constituents of the extract may be responsible for the antioxidant activity. Flavonoids or bioflavonoids are a ubiquitous group of polyphenolic substances which are present in most plants, concentrated in roots, seeds, fruit skin or peel, bark, and flowers (Amic *et al.,* 2007). Numerous studies have shown that flavonoids possess potent antioxidant activities capable of scavenging hydroxyl radicals, superoxide anions, and lipid peroxyl radicals (Amic *et al.,* 2007). Shahidi et al. (2013) attributed the pharmacological activities (anti-inflammatory, antiviral, antibacterial, antiulcer, antiosteoporotic, antiallergic, and antihepatotoxic actions) of flavonoids to their potent antioxidant activity.
In conclusion, *M. Oppositifolius* root demonstrated potent antioxidant effects which may be the rationale behind some of its folkloric uses and also may be responsible for some of its pharmacological effects.

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