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Identification and Quantification of Phytochemical Constituent and Antioxidant Activities of the Ethanolic Extract of *Mallotus oppositifolius* (Geiseler) Plant

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Abstract

Antioxidant studies, identification and quantification of phytochemical constistuents were carried out on the ethanolic extract of the leaves and bark of Mallotus oppositifolius (Geiseler) plant. Secondary metabolites such as alkaloids, flavonoids, tannins, saponins and phenolic were found to be present in significant quantities. The presence of these phytochemical constituents is responsible for the plants antioxidant property which was found to be 5.953 %. Gas chromatography-mass spectroscopy (GC-MS) was used for the identification and quantification of bioactive constituents in the extract. Twenty seven compounds were identified in M. oppositiofolius extract with Xanthoxylin (31.29 % peak area; molecular Formula; $C_{10}H_{12}O_4$ and retention time 11.887 minutes) having the highest concentration in the extract had been reported to increase melanin production that help in absorbing toxic drugs and chemicals as well as protecting the skin against skin cancer and deoxyribonucleic acid (DNA) damage.

1. Introduction

Plant products have recently become main scientific interest due to their numerous uses and applications. Medicinal plants are the richest bio-resource of drugs of traditional system of medicine, modern medicines, food supplements, pharmaceutical intermediates and chemical entities for synthetic drugs [1]. The knowledge of traditional medicine has been harness for a very long time in management and treatment of various ailments by humans. Presently, an average of seventy percent of humans is dependent upon medicines derived from plants and plant products [2].

The medicinal function of plants is derived from their possessing some chemical substances that produce physiological action on living tissues. The most important bioactive constituents of plants, alkaloids, tannins, flavonoids and phenolic compounds, have low toxicity, target specificity and are easily biodegradable when compared to active constituents of synthetic drugs [3].Plants have an overwhelming ability to synthesis secondary metabolites. These metabolites are the phytochemical constituents responsible for the bioactivity of medicinal plants. It is reported that about 12000 of these metabolites have been isolated and characterized and that this number represents a small fraction (less than 0.1) of the total [4].

Mallotus oppositifolius is a common shrubby weed in forest re-growths that reproduces from seeds. It belong the family of Euphorbiaceae with common name, 'Indian kamila', In Nigeria the

Mallotus oppositifolius plant is known locally as ‘‘Kafar Mutuwaa’’ by Hausas, ‘‘Ija’’ by Yorubas and‘‘Nne Okpo Kirinya’’ by Igbos [5]. *M. oppositifolius* twig is used as chewing sticks for cleaning the teeth; the stem as yam stakes, cold infusion of the leaves as tapeworm expellant and the crushed leaves are applied to eye inflammation during small pox attack [6]. In this study, the quantitative phytochemical composition and the antioxidant activities of the ethanol extract of the leaves with bark of *M. oppositifolius* (Geiseler) were carried out.

2. Methodology

2.1 Sample Extraction

A mixture of 50g of plant sample and 500 ml ethanol in a flask was incubated for 72 hours with intermittent shaking. The mixture was filtered with a muslin cloth into sterile glassware. Concentration of the filtrate was carried out at temperature below 60 ^{0}C with the aid of a water bath. The concentrated extract was weighed, wrapped in aluminum foil and stored at 4 ^{0}C prior to use.

2.2 Phytochemical and Antioxidant Analysis of *M. oppositifolius*.

Preparation of Sample Extract Solutions

From 250 g/ml of extract, other concentration (200, 150, 100, 50 ug/ml) were prepared using serial dilution.

2.3 Measurement of Free Radical Scavenging Activities Ethanolic Extract of Sample

The free radical scavenging activity of the plant extract was assayed using a stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) standard method [7, 8] with little modification. This was done by preparing a reaction mixture for each sample extract solution and standard solutions. The reaction mixtures, control and blank were allowed to incubate in the dark for 30 mins.

The absorbance of the reaction mixtures were measured using UV/Visible Spectrophotometer at 518 nm wavelength. The ability of the extracts to scavenge DPPH radical was calculated with the aid of the equation:

$$\%\ \text{Free Radical Scavenging Activity} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample/Standards}}{\text{Absorbance of control}} x100 \quad (1)$$

2.4 Determination of Total Phenolic Content

The amount of phenolics in extract was determined with Folin–Ciocalteu reagent according to the method of Singleton and Rossi [9] with slight modification using tannic acid as standard.

A 1.0 ml aliquot of Folin–Ciocalteu reagent was added to1.0 ml of 250 µg/ml extract solution in a test tube and the content of the test tube was mixed thoroughly. After 5 min, 15.0 ml of 20 % Na_2CO_3 was added and allowed to stand for 2 hours. The absorbance was measured at 760 nm using a UV-Visible Spectrophotometer (Jenway 6100, Dunmow, Essex, U.K). The total phenolic content was determined as mirogram of tannic acid equivalent (µg/TAE) using the equation obtained from the standard tannic acid calibration graph.

2.5 Determination of Alkaloids Content

The total alkaloid content was measured using the method described by Usunobun and Okolie [10]. Five (5) grams of the extract was weighed into a 250 ml beaker and 100 ml of 20 % acetic acid in ethanol was added, covered and left to stand for 2 hours. The solution was filtered and the filtrate was concentrated to one quarter of the original volume using a water bath. Concentrated

ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The solution was allowed to settle and the precipitate was collected by filtration and weighed.

$$\% \text{ Alkaloid} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \text{ x } 100 \quad (2)$$

2.6 Flavonoid Content Determination

The flavonoid content was determined on triplicate aliquots of the extract (1.5 g) [11]. Thirty-microlitre aliquot of the methanolic extract was used for flavonoid determination. The sample was diluted with 90 µl methanol. This was followed by the addition of 6 µL of 10 % Aluminum chloride, 6 µL of 1 mol/L sodium acetate and 170 µL of methanol. The absorbance was read at 415 nm after 30 min. Quercetin was used as a standard for calculating the flavonoid content (µgQe/g).

2.7 Estimation of Total Saponins Content

Total saponins content was determined by the method described by [12] based on vanillin-sulphuric acid colorimetric reaction with slight modifications. 250 µL of distilled water was added to 50 µL of plant extract. To this, about 250 µL of vanillin reagent (800 mg of vanillin in 10 ml of 99. 5 % ethanol) was added. Then 2.5 ml of 72 % sulphuric acid was added. The solution was mixed thoroughly and kept in a water bath at 60 ^{0}C for 10mins. After 10 mins, it was cooled in ice cold water and the absorbance was read at 570 nm. Standard saponin solutions (0-25 ppm) were prepared from saponin stock solution. The standard solutions were treated similarly as test samples. The values were expressed as part per million (ppm).

2.8 Quantitative Determination of Tannin

This was carried out by the method reported by [13] with slight modification. A 0.20 ml of sample was added to 20 ml of 50 % methanol and placed in a water bath at 77 ^{0}C – 80 ^{0}C for 1 hour with intermittent shaking. The extract was filtered using a double layered Whatman No.1 filter paper. Thereafter, 20 ml of distilled water, 2.5 ml Folin-Denis reagent and 10 ml 17 % Na_2CO_3 were added. The mixture was allowed to stand for 20 minutes. Standard tannic acids solutions were prepared in methanol and their absorbance as well as that of the samples was read after colour development on a UV/Visible spectrophotometer at a wavelength of 760 nm. Total tannin content was calculated from the calibration curve obtained.

2.9 Gas Chromatography-Mass Spectroscopy Analysis of *Mallotus oppositifolius*

2.9.1 Preparation of Extract

The ethanolic extract of the leaves was analyzed using GC-MS for the identification of the phytochemical compounds present. A solvent blank analysis was first conducted using 0.5 µL of absolute ethanol. Then 0.5 µL of the reconstituted ethanolic extract solution was employed for GC-MS analysis [14].

2.9.2 Gas Chromatography-Mass Spectroscopy Analysis

GC-MS analysis was carried out on a GC system comprising a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) instrument; Shimadzu GCMS-QP2010SE and the following conditions were employed: Column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100 % Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999 %) as carrier gas at a constant flow of 1ml/minute and a sample injection volume of 0.5 µl which was employed, injector temperature 250 ^{0}C; ion-source temperature 280 ^{0}C. The oven temperature was programmed from 110 ^{0}C (isothermal for 2 minutes), with an increase of 10 ^{0}C/minute, to 200 ^{0}C, then 5 ^{0}C/minute to 280 ^{0}C, ending with a 9 minutes isothermal at 280 ^{0}C.

Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da. Total run time was 21 min.

2.9.3 Identification of compounds

The compounds were then identified from the GC-MS peaks, using library data of the corresponding compounds. GC-MS was analyzed using electron impact ionization at 70 eV and data was evaluated using total ion count (TIC) for compound identification and quantification. Interpretation of mass spectrum of GC-MS was conducted using the database of National Institute Standard and Technology (NIST Version-Year, 2005). The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The spectrum of the unknown component was compared with the spectrum of the component stored in the NIST data library (version 2005). The name, molecular weight, molecular formula and structure of the components of the test material were determined. Measurement of peak areas and data processing were carried out by Turbo-Mass-OCPTVS-Demo SPL software.

3. Results and Discussion

Phytochemical analysis of ethanol extract of *M. oppositifolius* revealed the presence of five important phyto constituents tested viz; flavonoids, tannins, saponins and phenolic compounds and alkaloids. Saponins, flavonoids, and phenolic compounds were present in high concentration while tannins were present in low concentration. The ethanolic extract yielded the phytochemicals in Table 1.

Table 1: Quantitative composition of the alcoholic leaves and stems extracts of *Mallotus oppositifolius*

TPC (µg/ml)TAE	TFC (µg/ml)QE	TT C (µg/ml)	T A (%)	TS (ppm)
18.4905	15.7352	2.3077	14.4000	7.3861

Key: TPC: Total Phenolic Content; TFC: Total Flavonoid Content; TTC: Total Tannin Content; TA: Total Alkaloids; TS: Total Saponins.

The antioxidant activity was measured by the ability of the extract to scavenge DPPH free radical and was compared with the standard, ascorbic acid (Table 2). It was observed that scavenging activity of the alcoholic extract of *M. oppositifolius* caused an inhibition of 5.953% equivalent to that of 14.9912 µg/ml ascorbic acid

Table 2: Antioxidants of the Alcoholic Extract of *Mallotus oppositifolius*.

% Inhibition	Equivalent conc. of Ascorbic (µg/ml)
5.9530	14.9912

GC–MS Chromatogram of the Ethanolic extract of *Mallotus oppositifolius* (Fig. 1) shows twenty-seven peaks, indicating the presence of twenty-seven Phyto-compounds. The identification of the compounds was confirmed based on the peak, area, retention time and molecular formula. The constituent phytochemicals with their retention time (RT), peak area in Percentage (%), molecular formula (MF) and molecular weight (MW) are tabulated in Table 3.

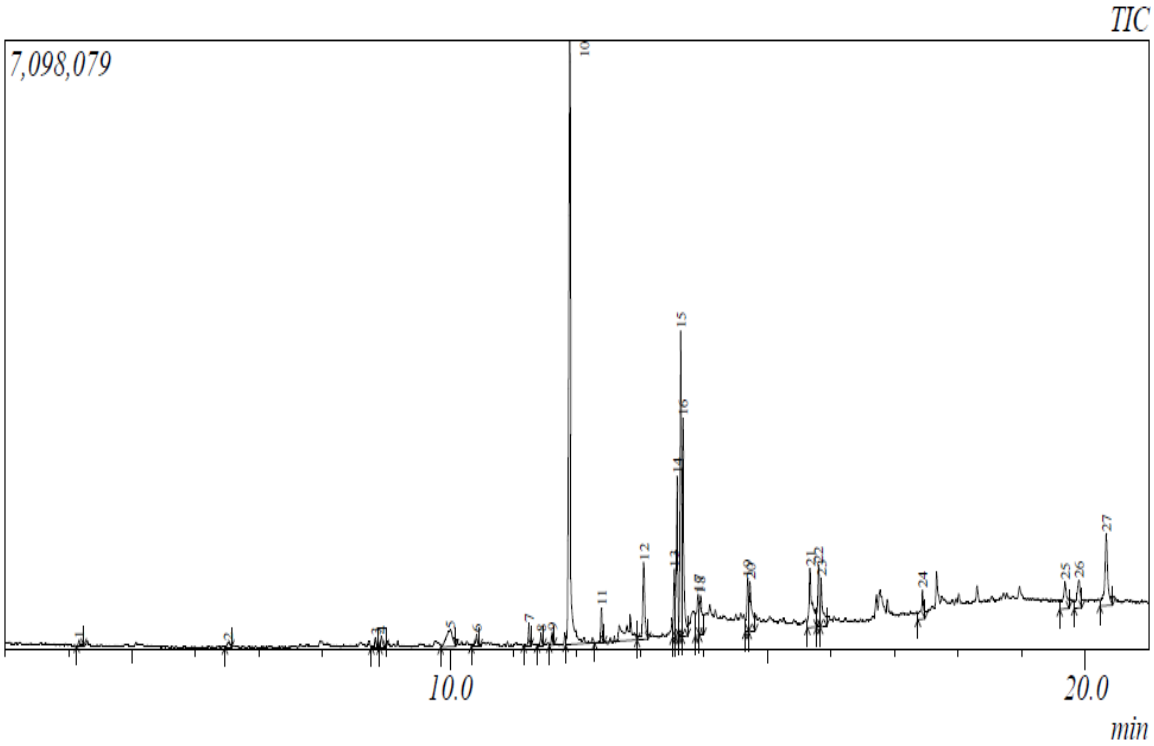

Fig 1: Chromatogram of *Mallotus oppositifolius*

Table 3: Phyto Compounds Identified in the Ethanolic Extract of *Mallotus oppositifolius* using GC-MS.

PEAK	COMPOUND NAME	RT	AREA	% AREA	MF	MW
1	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy	4.167	141862	0.26	$C_6H_8O_4$	144
2	Benzene, 2-methoxy-1,3,5-trimethyl-	6.512	185378	0.34	$C_{10}H_{14}O$	150
3	Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl	8.832	217508	0.39	$C_{15}H_{24}$	204
4	1H-3a,7-Methanoazulene, octahydro-1,9,9-trimethyl	8.9 26	227849	0.41	$C_{15}H_{24}$	204
5	3-O-Methyl-d-glucose	10.003	1473575	2.67	$C_7H_{14}O_6$	194
6	1-Naphthalenemethanol, decahydro-5-(5-hydroxy	10.424	377224	0.68	$C_{20}H_{34}O_2$	306
7	5-Caranol, trans,trans-(+)-	11.243	443194	0.80	$C_{10}H_{18}O$	154
8	2,3-Bis(1-methylallyl)pyrrolidine	11.436	270631	0.49	$C_{12}H_{21}N$	179
9	Pentafluorobenzoic acid, tridec-2-ynyl ester	11.607	252617	0.46	$C_{20}H_{23}F_5O_2$	390

10	Xanthoxylin	11.887	17262347	31.29	$C_{10}H_{12}O_4$	196
11	Hexadecanoic acid, methyl ester	12.389	650359	1.18	$C_{17}H_{34}O_2$	270
12	Aspidinol	13.053	2045911	3.71	$C_{12}H_{16}O_4$	224
13	7-Octadecenoic acid, methyl ester	13.534	1284683	2.33	$C_{19}H_{36}O_2$	296
14	3H-Pyrimido[5,4-c]1,2,5-oxadiazine-6,8(5H,7	13.577	3376779	6.12	$C_{12}H_{16}N_4O_3$	264
15	Phytol	13.634	4976350	9.02	$C_{20}H_{40}O$	296
16	Ethanone, 1-(3,4-dihydro-7-hydroxy-5-methoxy-	13.666	4774750	8.65	$C_{14}H_{18}O_4$	250
17	9,12-Octadecadienoic acid (Z,Z)-	13.907	1333671	2.42	$C_{18}H_{32}O_2$	280
18	Z,Z-4,16-Octadecadien-1-ol acetate	13.945	1291782	2.34	$C_{20}H_{36}O_2$	308
19	Hexadecanoi c acid, 1-(hydroxymethyl)-1,2-Ethanediyl	14.686	1133760	2.05	$C_{35}H_{68}O_5$	568
20	cis-11-Eicosenoic acid, methyl ester	14.726	1775998	3.22	$C_{21}H_{40}O_2$	324
21	Z,Z-4,16-Octadecadien-1-ol acetate	15.669	2496497	4.52	$C_{20}H_{36}O_2$	308
22	Erucic acid	15.802	1218351	2.21	$C_{22}H_{42}O_2$	338
23	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl	15.843	1427536	2.59	$C_{19}H_{38}O_4$	330
24	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-he	17.441	929705	1.69	$C_{30}H_{52}O$	428
25	Spiro[androst-5-ene-17,1'-cyclobutan]-2'-one, 3-	19.682	1338251	2.43	$C_{22}H_{32}O_2$	328
26	Ergostan-6-one, 3,25-bis(acetyloxy) -5-hydroxy .	19.900	1128609	2.05	$C_{32}H_{52}O_6$	532
27	gamma.-Sitosterol	20.328	3139373	5.69	$C_{29}H_{50}O$	414

The GC-MS analysis of the ethanolic extract of *Mallotus oppositifolius* led to the identification of twenty seven compounds (Table 3) with Xanthoxylin having the highest concentration in the

extract with percentage peak area of 31.29 %, retention time of 11.887 minutes and molecular formula $C_{10}H_{12}O_4$, followed by phytol (9.02 % peak area, R.T 13.634, and M.F $C_{20}H_{40}O$), Ethanone 1-(3,4-dihydro-7-hydroxy-5-methoxy (8.65 % peak area, R.T 13.666, and M.F $C_{14}H_{18}O_4$), 3H-Pyrimido [5,4-c]1,2,5-oxadiazine- (6.12 % peak area, R.T 13.577, and M.F $C_{12}H_{16}N_4O_3$), Gamma.-Sitosterol (5.69 % peak area, R.T 20.328, and MF $C_{29}H_{50}O$), Z,Z-4,16-Octadecadien-1-ol acetate (4.52 % peak area, R.T 15.669 ,and M.F $C_{20}H_{36}O_2$) while the remaining Compounds have constituted less than 4 % composition by peak area.

In contrast, Igwe *et al*. [15] reported the presence of nine bioactive compounds in the methanolic leaves extract of *M. oppositifolius,* with glutaconic anhydride having the highest concentration in the extract (40.19 %; $C_5H_4O_3$, R.T22.686 minutes), followed by 2-mercaptophenol with 18.23 % peak area, (R.T 22.068) and n-hexadecanoic acid (14.22 %; $C_{16}H_{32}O_2$), while the rest had less than 2 % composition by peak area.

The differences in the results observed in both research work in terms of the numbers of phytochemical compounds detected, could have occurred due to either the plant parts used (leaves only or with bark), or the solvent of extraction employed. This is supported by Akinnibosun *et al* [16] who reported that ethanol is the best medium of extraction for dried plant materials.

Among the identified phytochemicals, xanthoxylin was found to induce the production of melanin, Increases the number of dendrites tyrosinase, Skin pigmentation plays an important role in absorbing toxic drugs and chemicals as well as protecting the skin against skin cancer and DNA damage [17]. Loss of melanin in the epidermis can increase a person's risk in acquiring skin cancers and result in hypopigmentation such as vitiligo. Phytol which is an aditerpene alcohol that is a key precursor for syntheses of vitamin E and K1, is one of the compounds detected in this present study and has been reported to have antibacterial activities against *Staphylococcus aureus* by causing damage in cell membrane. These results in leakage of potassium ions from bacterial cells confirm the report of Byju *et al* [18]. Ethanone, a good flavoring agent, a strong antioxidants and anti-inflammatory agent that help reduce stress has a lethal effect against disease causing microbes [19]. Beta.-Sitosterol is an antioxidant, antimicrobial, antihelminthic and anti-neutralizing effects against viper and cobra venom [20, 21]. The antioxidant activity of the extract was comparable to that of standard ascorbic acid (Table 2). These findings provide scientific evidence to support traditional medicinal uses and indicate a promising potential for the development of antioxidant agent from *Mallotus oppositifolius*. It showed that ethanolic extract of *M. oppositifolius* possess significant antioxidant capacity which could be good scavenger of free radicals and invariably useful in the prevention of oxidative stress related diseases.

4. Conclusion

The phytochemical composition and antioxidant activity of the ethanolic extract of the leaves and bark of *Mallotus oppositifolius* plants has been investigated in this study. The various phytochemicals constituents identified in this study had been reported in literature to exhibit some levels of bioactivity such as antimicrobial, anti-asthma, anti-tumor, and anti-inflammatory. Therefore, *M. oppositifolius* is justifiably employed in the treatment of various diseases in tradition medicine practice.

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