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GC-MS Analysis, Antiglycation, Antioxidant and Anti-lipid Peroxidation Activities of *Harungana madagascariensis* Methanolic Stem Bark Extract

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Abstract

Plants' natural products make excellent lead for the development of new drugs used for prevention, management and treatment of communicable and non-communicable diseases. The specific aim of the study was to investigate the antioxidant and antiglycation activities of *Harungana madagascariensis* methanolic stem bark extract. The active compounds present in the plant were extracted using methanol by maceration and resulting extract was subjected to phytochemical screening, antioxidant and antiglycation analysis. The extract was fractioned on silica gel to isolate the antioxidant compounds domiciled in the extract and fractions were identified by bioautography. The *H. madagascariensis* methanolic stem bark extract showed the presence of tannins, alkaloids, saponins, steroids and flavonoids. From the antioxidant assays, the extract scavenged 2,2-diphenyl-1-picryhydrazyl (DPPH) and nitric oxide radicals, inhibited lipid peroxidation in a dose dependent manner showing IC_{50} higher than ascorbic acid while it inhibited albumin glycation with IC_{50} lower than ascorbic acid. From the 12 fractions obtained only 7 fractions showed antioxidant activity and quercetin, flavone, 6-hydroxyflavone, ferulic and o-coumaric acid were inferred. From the GC-MS analysis, fatty acids and their esters, terpenoids and steroids were identified such as Hexadecanoic acid ethyl ester (6.53 %), 9,12,15-Octadecatrienoic acid (5.92 %), 9,12,15-Octadecatrienoic acid methyl ester (0.95 %), vitamin E (1.22 %), phytol (3.87 %), squalene (1.66 %) and Pregn-5-en-3-ol, 21-bromo-20-methyl-(3.beta.)- (1.94 %). In conclusion, *H. madagascariensis* methanolic stem bark extract possessed antioxidant and antiglycation activities, thus, can be used for new drug development to reduce or combat oxidative stress related diseases in humans.

Keywords: GC-MS, Antioxidant, Antiglycation, Lipid peroxidation, Non-communicable diseases, *H. madagascariensis*, TLC

Introduction

Non-communicable diseases including cancer, diabetes, chronic respiratory diseases and cardiovascular diseases account for 29 % of mortality rate in Nigeria [1]. The common key factor responsible for the incidence of non-communicable diseases is oxidative stress which ensues from excessive production of free radicals in human body compared to the innate antioxidant system [2]. Free radicals can be produced endogenously by the body's normal metabolic and physiological processes [3] and exogenously by diet, radiation and other man made processes [4]. Contributing to this stress is glycation process. Glycation is a non-enzymatic reaction involving sugar and other biomolecules like proteins, lipids and nucleic acid which forms advanced glycation end products as a consequence of chronic hyperglycemia and progressed oxidative stress [5,6]. Oxidative stress and glycation contribute greatly to the progression of diabetes and diabetic complications like neuropathy, retinopathy, nephropathy and cardiovascular disease [7,8]. The occurrence of oxidative stress can be prevented by antioxidants.

Antioxidants are reducing agents that donate electrons to free radicals which convert them to harmless molecules [9]. They are equipped to quench, scavenge or neutralize free radicals generated in the body in order to prevent oxidative stress. The body's antioxidant system when overwhelmed can be cushioned by

exogenous sources obtained directly from diet, as supplements or from medicinal plants. Common dietary antioxidants are vitamin C, E and carotenoids. However, medicinal plants besides their primary nutrients, contain large amounts of secondary metabolites with antioxidant properties [10]. Thus, intensive research into plants to search for natural compounds with both antioxidant and antiglycation activities for the effective treatment and management of various human diseases. Since time immemorial, man has relied heavily on plants for various human needs including medicines. In recent times, man's interest in plants for therapeutic purpose has not dissipated despite advanced and sophisticated therapeutic measures by orthodox medicine [11]. The interest in the use of medicinal plants may be due to their perceived safety, availability and accessibility relevant to the well-being of a significant proportion of world's population [12,13]. Plants being a store of bioactive compounds with varied functional groups and distinct specialized physiological importance are being exploited by researchers to validate their ethnobotanical use and develop new drugs with multi-therapeutic functions.

Harungana madagascariensis (HM) is commonly called "amuje" among Yoruba tribe in Nigeria. The aqueous root decoction of the plant is employed in the treatment of liver and kidney diseases, dysentery, bleeding piles, trypanosomiasis, fever, cold and cough; the plant exudates are used to cure acute enteritis, scabies and jaundice while a decoction of the stem bark is used as hematinics and have been explored in Jubi formulation, used to restore pack cell volume (PCV) and hemoglobin level [14]. A decoction of the stem bark is used in the management of diabetes [15-17]. The bark is also used in the treatment of hepatitis, malaria, river blindness, asthma, dysmenorrhea, ulcer, toothache, nephrosis and gastrointestinal complications [18,19]. In Cameroon, the leaves and seed oil of *H. madagascariensis* are used to treat typhoid fever and malaria [20]. This study was conducted to investigate the antioxidant and antiglycation activities of *H. madagascariensis* methanolic stem bark extract.

Materials and methods

Sample collection

H. madagascariensis stem bark was purchased from a local herbal market (Mushin) in Lagos, Nigeria on 13th October, 2021. The test plant was identified and deposited in the University of Lagos Herbarium with the number LUH 8548.

Preparation and extraction of plant materials

The plant materials were cleaned with water to remove all contaminants, air dry for 4 weeks, crushed and ground to coarse form with a manual blender. Two hundred g of *H. madagascariensis* were weighed and extracted with methanol by maceration for 72 h. The mixture was filtered and the filtrate concentrated to dryness using a rotary evaporator to obtain a yield of 12.5 %.

Phytochemical screening

The presence of phytochemicals such as saponins, flavonoids, alkaloids, steroids, glycosides, carbohydrates and tannins in the methanol stem bark extract of *H. madagascariensis* was determined using the standard procedures described by Evans [21]; Harbone [22]; Sofowora [23].

Determination of total phenolic content

The total phenolic content of the extract was determined using the method of Chun *et al.* [24]. Folin-Ciocalteu's reagent (1 mL) was added to the sample (1 mL, 1.0 mg/mL) and mixed thoroughly. To this mixture, 4 mL of sodium carbonate (75 g/L) and 10 mL of distilled water were added and thoroughly mixed. The mixture was allowed to stand for 90 min at room temperature and absorbance of the reaction mixture was taken using Thermo Scientific Genesys 10S UV-Vis Spectrophotometer at 760 nm. The total phenolic content was extrapolated using a calibration curve ($R^2 = 0.9699$) for gallic acid. The results were expressed as the gallic acid equivalent per gram of dry weight of extract (mg of GAE/g of extract). All samples were analyzed in triplicate and values were expressed as mean ± SD.

Determination of total flavonoid content

The total flavonoid content of the extract was measured by the aluminum chloride colorimetric assay described by Chang *et al*. [25]. The plant extract (0.5 mL, 1.0 mg/mL) was mixed with methanol (1.5 mL), Aluminium chloride (0.1 mL, 10 %), sodium acetate (0.1 mL, 1 M) and distilled water (2.8 mL). The reaction mixture was left for 30 min at room temperature and the absorbance was read at 415 nm. The total flavonoid content was calculated using a calibration curve ($R^2 = 0.9961$) for quercetin. The results were

expressed as the quercetin equivalent per g of dry weight of extract (mg of QAE/g of extract). All samples were analyzed in triplicate and values were expressed as mean ± SD.

Total antioxidant capacity

Total antioxidant capacity of the plant extract was assessed using the phosphomolybdenum method, an assay based on the reduction of Mo (V1) to Mo (V) by the extract and the subsequent formation of green phosphate/Mo (V) complex at acidic pH by adding 4 mL reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate, 4 mM Ammonium molybdate. The tubes were incubated in the water bath at 95 °C for 90 min. After the samples had been cooled to room temperature, the absorbance of the mixture was measured at 695 nm against blank. The phosphomolybdenum method is quantitative, since, the total antioxidant activity is expressed as the number of equivalents of ascorbic acid [26].

DPPH free radical scavenging activity

The free radical scavenging activity of both extracts was measured as a decrease in the absorbance of methanol solution of DPPH. Equal volumes of DPPH solution (0.033 g/L in methanol) were added to extract solution at different concentrations (20 - 100 µg/mL). After 30 min, absorbance was measured using Thermo Scientific Genesys 10S UV-Vis Spectrometerat 517 nm and compared with standard ascorbic acid [27].

Nitric oxide scavenging assay

The ability of *H. madagascariensis* bark extract to scavenge nitric oxide obtained from sodium nitroprusside was performed according to the method of Ogundare *et al*. [28]. The plant extract (20 - 100 µg/mL) or ascorbic acid (0.5 mL) was separately mixed with 2.5 mL of 0.01 M sodium nitroprusside in phosphate buffer (pH 7.4) and left at room temperature for 2 h 30 min. An aliquot of the reaction mixture was combined with equal volume of Griess reagent and its absorbance taken at 540 nm against related solution of blank (plant extract or ascorbic acid and equal volume of distilled water). The control contained the same reaction mixture in the absence of extract but distilled water. Absorbance was measured using Thermo Scientific Genesys 10S UV-Vis Spectrometer at 540 nm. All the reactions were carried out in triplicate.

***In-vitro* anti-lipid peroxidation assay**

Freshly excised goat liver (2 g) was processed to obtain 10 % homogenate in cold phosphate-buffered saline, pH 7.4, and centrifuged to obtain clear tissue homogenate. Five hundred µL of different concentrations of the extracts were added to 3 mL tissue homogenate. Then, the lipid peroxidation was initiated by adding 100 µL of 15 mM ferrous sulphate solution. After 30 min, 100 µL of the reaction mixture in various test tubes was taken in a tube containing 1.5 mL of 10 % trichloro acetic acid (TCA). After 10 min, the tubes were centrifuged and mixed with 1.5 mL of 0.67 % thiobarbituric acid (TBA) in 50 % acetic acid. The resulting supernatant obtained after centrifuging at 800 g for 15 min was collected into a clean test tube and was heated in a boiling water bath, cooled and the intensity of the pink-colored complex formed was measured at 535 nm [29].

Determination of antiglycation activity

Bovine serum albumin (500 µL) was incubated with glucose (400 µL, 500 mM), extract (100 µL) at various concentrations and phosphate buffer saline (pH 7.4, 100 µL). The reaction was allowed to proceed for 24 h at 60 ℃ and terminated by adding 10 µL of 100 % TCA and kept at 4 ℃ for 10 min. The mixture was centrifuged for 4 min at 13,000 rpm and the precipitate obtained was re-dissolved in 2.5 mL alkaline phosphate buffer saline (pH 10). The resulting mixture obtained was quantified for the relative amount of glycated BSA using a Jenway 6,270 Spectroflourimeter. The excitation and emission wavelength used were at 370 and 440 nm, respectively [30].

Detection of antioxidant compounds by TLC bioautography

H. madagscariensis methanolic extract was fractioned on a silica gel (60 - 200 mm) column using the wet packing method. The sample was applied using the dry loading method and compounds were eluted using solvents of increasing polarity, that is, hexane, ethyl acetate and methanol. The fractions obtained were profiled on TLC to ascertain their purity using R_f values. The fractions with antioxidant activity were detected using the TLC bioautography as described by Dewanjee *et al.* [31]. The fractions were spotted on TLC plates and sprayed with 0.2 % DPPH in methanol. The plates were observed in daylight after 30 min for yellow or cream spots against purple background. Phenolic compounds present in the extract was

identified using thin layer chromatography as described by Omotayo *et al.* [26]. The R_f values of the various spots obtained using 2 different solvent systems were compared to standard flavonoids and phenolic acids adopted in the work of Medic-Sari *et al.* [32].

System 1: n-hexane: ethyl acetate: acetic acid (31:14:5)

System 2: toluene: ethyl acetate: acetic acid (36:12:5)

GC-MS analysis *H. madagscariensis* methanolic extracts

The *H. madagscariensis* methanolic extract was subjected to GC\MS analysis, to characterizing its various volatile components. The gas chromatograph (model: 7890A (GA), Aligent Technologies interfaced with Mass Selective Detector model: 5975C (MSD; electron ionization (70 V) with an ion source temperature at 250 ℃; was equipped with an HP-5 capillary column (30 mm × 0.25 mm × 0.320 µm). The carrier gas was highly pure helium (99.9 % purity) with a flow rate of 1 mL/min, the injector mode-split (1:60), and the injection volume of 1 µL. The oven temperature was at 100 ℃ (0.5 min) and ramped to 140 ℃ at 20 ℃/min holding for 1 min, then ramped to 280 ℃ (20 min) at the rate of 11 ℃/min. The identification of the components was based on the comparison of their mass spectra with those of the NIST-Wiley 2008 library.

Statistical analysis

All data were presented as the mean of 3 separate experiments and error bars are displayed with standard error. The IC_{50} value was determined from linear regression analysis using Microsoft excel 2013.

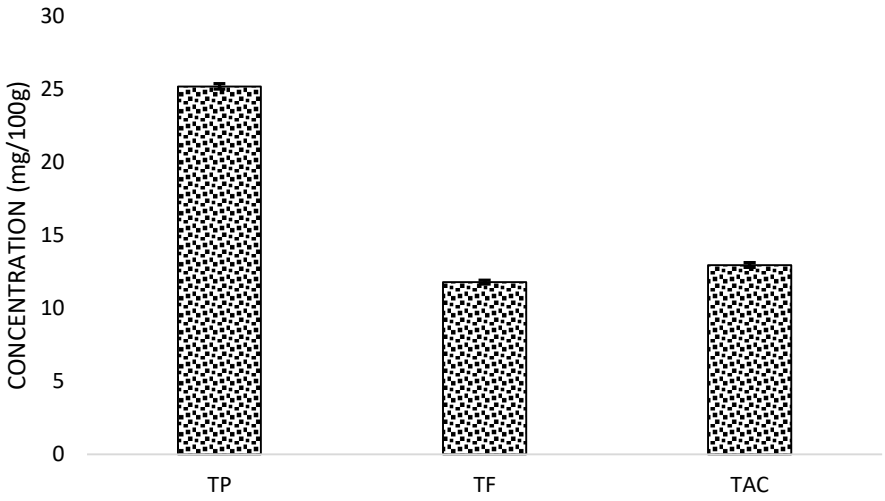
Results and discussion

Phytochemicals are plants' secondary metabolites that are responsible for their medicinal properties. In this study, saponins, alkaloids, steroids, cardiac glycosides, phenol and flavonoid were detected in the methanolic stem bark extract of *H. madagscariensis* while tannin was not detected (**Table 1**). Alkaloids have been widely reported to possess diverse healing properties including anti-diabetic and antioxidant properties [33,34]. Cardiac glycosides best known for their cardiotonic properties [35] also improve glucose tolerance and ameliorate diabetic complications [36]. Saponins are known to possess many medicinal properties including anti-inflammatory, venotonic and anti-oedematous activities [37,38]. They also help to reduce blood cholesterol levels, stimulate the immune system and fight cancer [39]. Phenolic compounds including flavonoids like flavones, flavonols, isoflavones, flavanones and chalcones, are known popularly as nature's tender drugs with numerous biological and pharmacological activities such as prevention of cancer, neurodegenerative disease, cardiovascular disease and diabetes [40]. They occur as a major constituent in food of plant origin and possess numerous biological/and pharmacological activities like antiviral, antifungal, antioxidant, anti-inflammatory, antiallergenic, antithrombic, anticarcinogenic, hepatoprotective and cytotoxic activities [41-43]. The presence of flavonoids in crude plant extracts may be responsible for their ability to act as antioxidant agents.

Table 1 Phytochemical constituents of *H. madagscariensis*.

Phytochemicals	*H. madagscariensis*
Alkaloids	+
Saponins	+
Flavonoids	+
Tannins	-
Steroids	+
Phenols	+
Reducing sugars	+
Cardiac glycosides	+

Key: + means detected while – means not detected



Key: TP - total phenolics, TF - total flavonoids and TAC - total antioxidant capacity.
Figure 1 Total phenol, flavonoid and antioxidant capacity of *H. madagscariensis* stem extracts.

The antioxidant activity of *H. madagscariensis* stem bark extracts in this study was determined by assessing their ability to scavenge DPPH and NO radicals. The ability of *H. madagscariensis* to scavenge DPPH and NO radicals was presented in **Figures 2** and **3**, respectively. The extract scavenged DPPH and NO radicals in a concentration-dependent manner with IC_{50} of 63.63 ± 2.42 and 70.31 ± 0.91 µg/mL, respectively. The IC_{50} value obtained for DPPH in this study was higher than the results obtained by Anita *et al.* [44]. This may be connected to the nature of the extract (defatted extract) and the concentration of DPPH employed in their study.

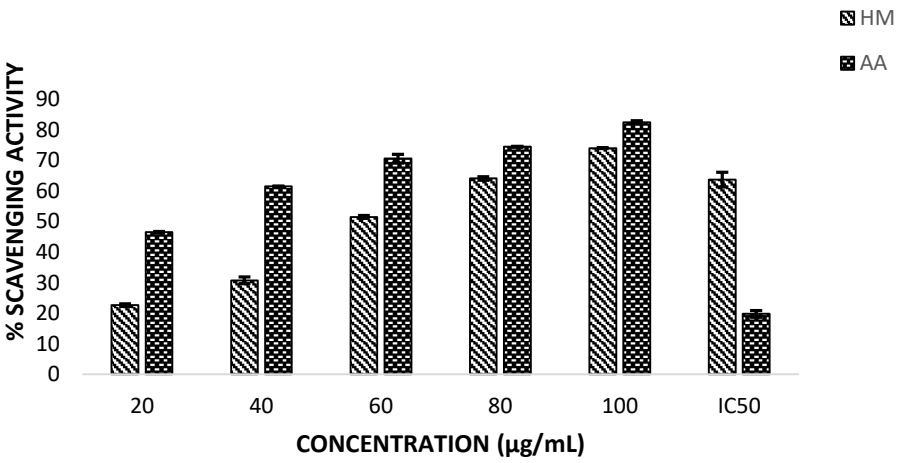


Figure 2 DPPH radical scavenging activity of *H. madagascariensis* methanol stem bark extract.

Nitric oxide is generated *in-vivo* by macrophages, neurons and endothelial cells from the amino acid L- arginine. It is a chemical mediator involved in the regulation of several physiological processes, however, overproduction of nitric oxide and its related reactive nitrogen species are associated with the progression of several diseases like diabetic complications, cancer, Alzheimer's, arthritis multiple sclerosis and ulcerative colitis [45,46]. The IC_{50} for nitric oxide obtained in this study was lower than that obtained in the study of Afieroho and Afieroho [47] who utilized the chloroform extract of *H. madacascariensis* seed oil.

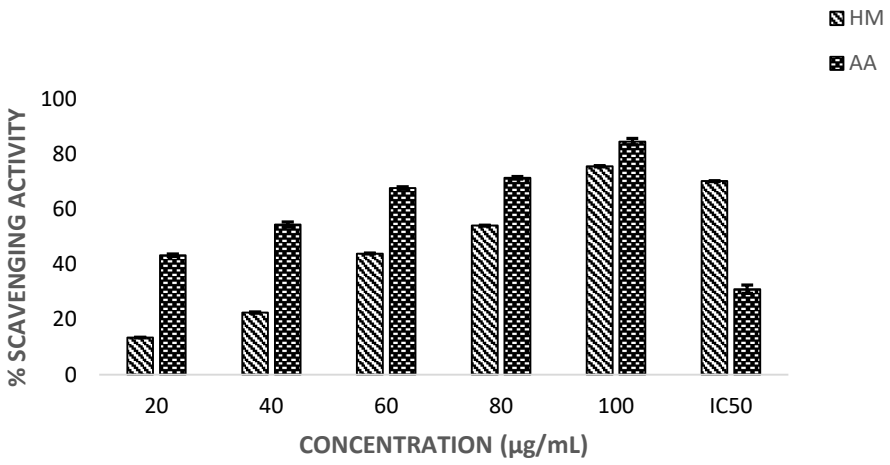
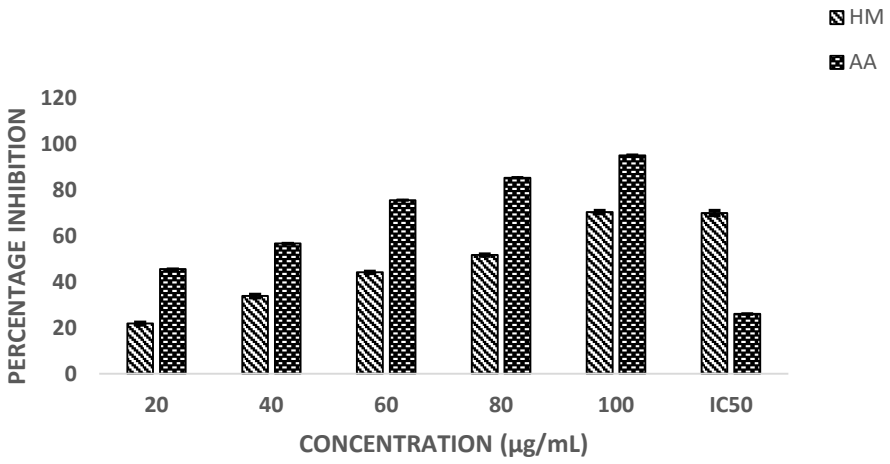


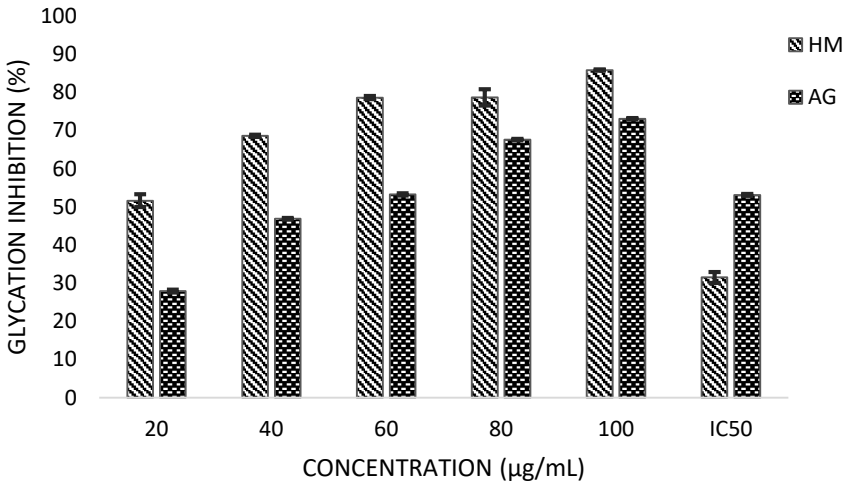
Figure 3 Nitric oxide scavenging activity of *H. madagascariensis* methanol stem bark extract.

Lipid peroxidation is widely recognized as a primary toxicology event caused by the deleterious effect of free radicals from a variety of sources including organic hydroperoxides, redox cycling compounds and ion-containing compounds [48]. **Figure 4** shows the percentage inhibition of lipid peroxidation activities of the extract of *H. madagscariensis.* The IC_{50} value of 70.02 ± 1.17 and 26.03 ± 0.19 µg/mL was obtained for methanolic stem bark extract of *H. madagscariensis* and ascorbic acid, respectively. The IC_{50} obtained in the entire test for methanolic stem bark extract of *H. madagscariensis* was higher than ascorbic acid which means that ascorbic acid is a better antioxidant because the lower the IC_{50}, the more active the compound is. However, this is expected because ascorbic acid is a pure compound while methanolic stem bark extract of *H. madagscariensis* is a mixture of compounds which needs to be purified to obtain pure drug.



HM: *H. madagscariensis*; AA: Ascorbic acid
Figure 4 Antilipid peroxidation activity of *H. madagascariensis* methanol stem bark extract.

The antiglycation activity of methanolic stem bark extract of *H. madagscariensis* and aminiguanidine was dependent on the concentration giving IC_{50} values of 31.53 ± 1.43 and 53.21 ± 0.23 µg/mL, respectively (**Figure 5**).



HM: *H. madagscariensis* ; AG: Aminoguanidine
Figure 5 Antiglycation activity of *H. madagascariensis* methanol stem bark extract.

The separation of the plant extract on silica gel produced 31 fractions with varied colors. Profiling of these fractions on TLC showed fractions with similar R_f values which informed the combination to produce twelve fractions. Fractions with good antioxidant activity were detected by spraying spots of these fractions on TLC plates with 2,2-diphenyl-1-picrylhydrazyl (DPPH, 0.2 %) in methanol. The development of a yellow spot against purple background indicates the presence of an antioxidant compound. All the fractions showed good antioxidant activity except fractions 5, 7, 11 and 12 evident in their white color observed against the purple background as shown in **Figure 6**. The phenolic compounds in the methanolic stem bark extract of *H. madagscariensis* were partially identified by using TLC. Compounds like flavone, 3'-hydroxyflavone, 3,7-dihydroxyflavone, quercetin, kaempferol, naringenin, morin, caffeic acid, 6-hydroxyflavone, o-coumaric acid and p-coumaric acid were inferred. The detection of these phenolic compounds in the fractions may be responsible for the antioxidant activity of methanolic stem bark extract of *H. madagscariensis* observed in this study.

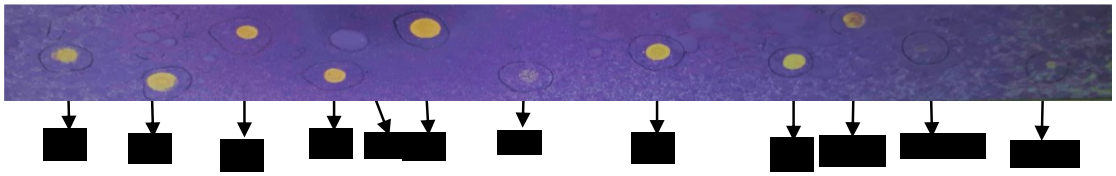


Figure 6 TLC bioautography of *H. madagascariensis* methanol stem bark fractions.

Table 2 Identification of phenolic compounds in *H. madagascariensis* methanol fractions using TLC.

Fraction	No. of spot	R_f	Inferred compound
1	1	0.92	Flavone
2	2	0.83, 0.56	Unidentified
3	2	0.80, 0.50	3'-hydroxyflavone, 3,7-dihydroxyflavone
4	2	0.75, 0.50	Unidentified, kaempferol
5	1	0.51	O-coumaric acid or p-coumaric acid
6	1	0.27	Quercetin
7	1	0.24	Unidentified
8	2	0.58, 0.35	Naringenin, unidentified
9	1	0.14	Morin
10	2	0.30, 0.61	Caffeic acid, 6-hydroxy flavone
11	1	0.59	Unidentified
12	1	0.65	Unidentified

Table 3 Constituents identified in *H. madagascariensis* methanol stem bark extracts by GC-MS.

S/N	Retention time	Area (%)	Compound name
1	5.305	0.36	2-Butenoic acid, 2-methyl-, (z)-
2	11.779	0.27	2(4H)-Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-
3	15.787	6.53	Hexadecanoic acid, ethyl ester
4	16.636	0.95	9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)-
5	16.769	3.87	Phytol
6	17.098	5.92	9,12,15-Octadecatrienoic acid, (Z, Z,Z)-
7	17.317	1.32	Octadecanoic acid, ethyl ester
8	18.617	0.61	9-Octadecenamide, (Z)-
9	18.778	0.70	Hexanedioic acid, bis(Ethylhexyl) ester
10	18.975	0.09	1,2-15,16-Diepoxyhexadecane
11	20.037	0.21	Nonadecanoic acid, ethyl ester
12	20.257	0.08	11,13-Dimethyl-12-tetradecen-1-ol acetate
13	21.481	1.66	Squalene
14	21.608	0.12	3-Cyclohexene-1-methanol, .alpha., 4-dimethyl-alpha.-(4-methyl-3-pentenyl)-, [R-R*,R*)]-
15	21.839	0.19	Octadecane, 1-bromo-
16	22.203	0.26	.delta.-Tocopherol
17	22.798	0.47	.gamma.-Tocopherol
18	23.289	1.22	Vitamin E
19	23.988	0.59	Campesterol
20	24.207	0.31	Stigmasterol
21	24.675	1.94	Pregn-5-en-3-ol, 21-bromo-20-methy l-, (3.beta.)-
22	24.981	0.69	.beta.-Amyrone
23	25.160	0.26	Lup-20(29)-en-3-one
24	26.304	0.50	Lup-20(29)-en-3-ol, acetate, (3.beta.)-
25	26.696	0.24	Phytyl dodecanoate

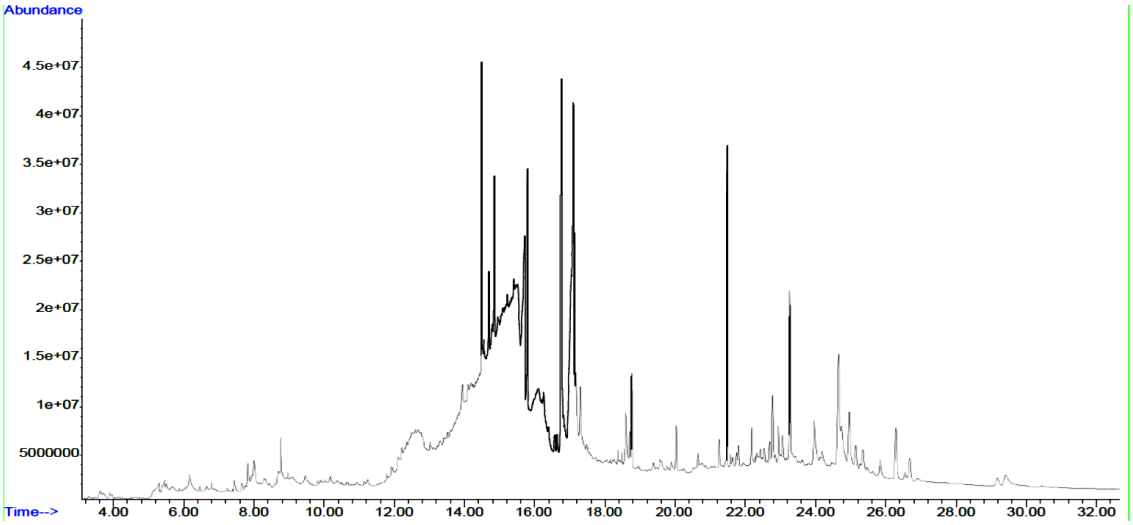


Figure 7 GC-MS total ion chromatograms (TIC) of chemical constituents of the methanol stem bark extracts of *H. madagascariensis*.

GC-MS analysis is used widely in the field of natural products to separate the volatile components in plant extracts or samples and presents a characteristic spectral output used to identify each component. From GC-MS results obtained in this study, 117 compounds detected, however compounds with qual number of ≥ 90 were selected and listed in **Table 3**. From the Table, 25 compounds were listed and account for 29.36 % of the plant extract. Identified compounds include fatty acids and their esters, terpenoids and steroids.

9,12,15-Octadecatrienoic acid, (Z, Z,Z)- or linolenic acid is an essential fatty acid not produced in humans but usually found in plants and other animals [49]. It has been reported to be beneficial in the prevention and management of many chronic diseases including coronary heart disease [50]. Araújo *et al.* [51] also reported the antimicrobial, anti-inflammatory, hepatoprotective, antihistaminic, antieczemic, antioxidant, hypocholesterolemic, cancer preventive, antiarthritic and anticoronary activity of fatty acids like palmitic acid (hexadecanoic acid and hexadecaonoic acid ethyl ester), linolenic (docosatetraenoic acid and octadecatrienoic acid). 9-Octadecenamide, (Z)- is an amide reported to possess antimicrobial activity [52]. Vitamin E or tocopherol and its various biological forms including gamma and delta tocopherol are predominantly known for their antioxidant properties. Phytol is a terpenoid used as a precursor for synthesis of fat soluble vitamins E and K1 [53] with strong antioxidant and possess anti-inflammatory activity [54]. Lup-20(29)-en-3-ol, acetate, (3β)-, a triterpenoid also known as lupeol acetate possess antimicrobial, anti-inflammatory, antimalarial, antituberculosis, antinociceptive and anticancer activity against breast cancer cells MCF-7 [55,56]. β-Amyrone has been reported to exhibit antifungal, anti-α-glucosidase inhibitory activity and moderate anti-acetylcholinesterase (AChE) activity [57]. It is therefore not unlikely that the presence of these phytochemicals in *H. madagascariensis* methanolic stem bark extract may play major roles in the biological activities and pharmacological properties of the plant.

Conclusions

The results reveal that the methanol extract of *H. madagascariensis* has strong antiglycation and radical scavenging activity due to the presence of various chemical components with reported bioactivities, thus, can be used for new drug development to reduce or combat oxidative stress related diseases in human.

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