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Evaluation of some antioxidant indices of the fresh flowers of *Harungana madagascariensis* Lam. Ex Poiret (Hypericaceae)

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Abstract
This study reports some antioxidant indices: free radical scavenging (FRS), total phenolics content (TPC) and total flavonoids content (TFC) of the fresh flowers of *Harungana madagascariensis* a plant widely used in folklore medicine. The crude absolute ethanol (FLE) extract was obtained by cold maceration and then partitioned with dichloromethane to obtain the non-polar dichloromethane (FLD) and the polar aqueous (FLA) fractions. The antioxidant properties FRS, TPC and TFC were respectively evaluated *in vitro* using the standard the diphenylpicryl hydrazine (DPPH), Folin Ciocateau and Aluminium chloride spectrophotometric assays. Phytochemical screening was done using standard reagents. At 100 µg/mL test concentration, the FLE, FLA, and FLD exhibited a significantly different ($p<0.05$) FRS activity. The trend in free radical scavenging property (IC_{50} (µg/ml)): FLD (16.80) >Ascorbic acid (12.17) >FLE (11.36) > FLA (7.36). Triterpenoids, cardiac glycosides, Phenolics like flavonoids, and carbohydrate derivatives were present in the flowers while anthraquinones, alkaloids, saponins and cyanogenic glycosides were however absent. This study is reporting for the first time the antioxidant and phytochemical properties of the flowers of *H. madagascariensis* a plant widely used in ethno-medicine.

Keywords: *Harungana madagascariensis*, flowers, oxidative stress, radical scavengers

Introduction
Several plant-derived natural compounds otherwise known as phytochemicals are known to act as radical scavengers [1]. The substances which neutralize the free radicals or their actions are known as antioxidants and play a vital role in mitigation of damages to cells, proteins, lipids and DNA by the oxidative stress caused by these radicals. This oxidative damage is considered to play a causative role in aging and several diseases of degeneration [1-2]. *Harungana madagascariensis* (Hypericaceae) is an evergreen bushy tree. It usually grows up to 12 meters tall. Various morphological parts are widely used in Africa traditional medicine practice. It used in the treatment of dysentery, diarrhea, anemia, typhoid and heart ailments are documented [3-5]. The roots and bark are used to treat gonorrhea, leprosy, hemorrhoids and to facilitate childbirth [3] while the sap is used for the treatment of skin diseases and for wounds [6]. The Juice from the leaves and stem bark are used to stop bleeding during child birth [7-8]. The fruits (raw) have sweet flavour, they are eaten as a snack, especially by children [9]. The fruits are also used in local cooking and are fermented to produce cider like drink which taken on an empty stomach which may sometimes cause vomiting. There are literatures on the scientific evaluation of the antibacterial [10-11], antiprotozoan [12] and antioxidant [13-15] activities of fruits, stem bark, roots and leaves of *H. madagascariensis*. The phytochemical composition of different morphological parts [10-12, 16] except the flowers have also been reported. Also, aside earlier report on the nitric oxide scavenging activity [14] and total phenolic and flavonoid content and DPPH radical scavenging activity [15] of the fruits, similar literature reports on the flower are not available. As a follow-up to these earlier reports [14-15], this present study is aimed at evaluating the flowers of *Harungana madagascariensis* for phytochemical constituents and free radical scavenging activity.

Materials and methods
Sample collection and authentication
Fresh flowers of *Harungana madagascariensis* used for this study were collected from the Medicinal Garden, Faculty of Pharmaceutical Sciences, University of Port Harcourt, Rivers State and authenticated by taxonomist in the herbarium of the Plant Science and

Biotechnology Department, University of Port Harcourt with herbarium number: UPH/P/080; UPH/V/1,219.

Reagents, solvents, equipment and apparatus used

The following analytical grade reagents and/or solvents were used in the course of carrying out the research work: dichloromethane, absolute ethanol, ferric chloride, distilled water, concentrated hydrochloric acid, Keddes reagent, picric acid, Molisch reagent, Fehling solution A and B, ammonia solution, concentrated sulphuric acid, acetic anhydride, magnesium fillings, glacial acetic acid, 10% hydrochloric acid, 10% sulphuric acid, sodium hydroxide, methanol, folin Ciocateau reagent, 10% aluminium chloride, tannic acid, quercetin. Ascorbic acid, sodium carbonate, sodium hydroxide and diphenyl picryl hydrazine (DPPH). Equipment used include: rotary evaporator, UV-Visible spectrophotometer (Novel series N4).

Extraction method

Fresh samples of the flowers (133.6 g) were pulverized with quenching in absolute ethanol and immediately extracted exhaustively with the same absolute ethanol by cold maceration for 8 days with fresh replacement every 48 hours. The ethanol extract obtained was concentrated using the rotary evaporator at temperature of 45°C and partitioned with dichloromethane using separating funnel to afford the non-polar dichloromethane and polar aqueous fractions. The extracts and fractions (the flower crude ethanol extract (FLE), flower aqueous fraction (FLA), and flower dichloromethane fraction (FLD)) were kept and used subsequently in this study.

Phytochemical Screening

Preliminary phytochemical tests for the presence of: alkaloids(Dragendorff's test), anthraquinones (Borntrager's test), triterpenoids(Liebermann-Buchard and Salkowski's tests), cardiac glycosides (Kedde(cardenolide nucleus) and Keller-Killiani's(Deoxysugar's) tests), phenolics($FeCl_3$, Phlobatannin's and Shinoda's(flavonoids) tests), saponins (Frothing test), carbohydrates(Molisch and Fehlings tests), cyanogenic glycosides(alkaline picrate paper's test) were carried out on the fresh flowers using standard phytochemical screening reagents [17-18]

Preliminary quantitative Free radical scavenging assay method

This was done using the DPPH spectrophotometric assay method[19]. Briefly, stock solutions (100 µg/ mL) of the crude ethanol extract (FLE) and the various fractions (FLD, FLA) obtained from the crude ethanol extract were prepared in methanol. DPPH (0.05% w/v in methanol) solution was also prepared. Using a pipette, 5 mL aliquots of the 0.05% w/v DPPH stock solution was added respectively to each concentration of the various samples solutions (5 mL), mixed and allowed to stand for 30 minutes in a dark environment. The absorbance of the various concentrations of the test samples were measured using a UV/Visible spectrophotometer (NOVEL 4S)) in duplicates at 517 nm. Methanol was used as reagent blank while methanol: DPPH stock solution (1:1 v/v) was used as negative control. Antioxidant activity was recorded as the percentage reduction in the absorbance of DPPH by either the test samples or standard.

% inhibition = [(Acontrol-Asample) / Acontrol] × 100
Where Acontrol= absorbance of negative control and
Asample = absorbance of sample

Concentration dependent free radical scavenging assay and determination of median inhibition concentration (IC_{50})

Based on the results from the preliminary quantitative Free radical scavenging assay above serial concentrations (8.0 -100 µg/mL) of the extracts and fractions were prepared. Each of these concentrations were separately evaluated for free radical scavenging activity as outlined above to establish a concentration dependent free radical scavenging profile. The half maximal inhibitory concentration (IC_{50}) which is the concentration of the sample which produces 50% of reduction in absorbance value was then extrapolated from the regression plot of percentage inhibition of DPPH activity against concentration. As a reference standard for comparison, ascorbic acid at concentration 0.080-0.005 mg/mL was similarly evaluated.

Determination of the total phenolic content

This Folin-Ciocalteau method[20] with modification as reported earlier [15] was used with the total phenolic content expressed as milligram tannic acid equivalent per gram of fresh flowers. Briefly, tannic acid stock solution (1 mg/ml) in methanol was prepared. Aliquots: 0.05, 0.10, 0.20, 0.40 and 0.60 mL of this stock solution were separately transferred into their respective 10 ml volumetric flask labeled R1-R5. To the content of each of these volumetric flasks R1-R5 was separately added 800 µL of Folin-Ciocalteau reagent followed by 2 mL of aqueous sodium carbonate (75 g/L) and the volume adjusted to 10 mL mark with distilled water. This was thereafter left to incubate in the dark for 2 hours for complete reaction. The absorbance of each of the reaction solutions in R1-R5 was taken at 765 nm and the calibration curve obtained from a plot of absorbance against concentration using Microsoft Excel 2013 package and the regression equation noted. For the *H. madagascariensis* fresh flowers, 1.004 g of the fresh flowers was exhaustively extracted with methanol and the volume of the methanol filtrate adjusted to 50 ml in a 50 ml volumetric flask. A 0.5 ml aliquot of this resulting solution was then transferred to a 10 mL volumetric flask followed by the addition of 0.8 mL of Folin-Ciocalteau reagent, 2 mL of aqueous sodium carbonate (75 g/L) and the volume adjusted to 10 mL mark with distilled water. This was thereafter left to incubate in the dark for 2 hours for complete reaction. The absorbance of the reaction solutions was taken at 765 nm and the total phenolic content TPC calculated from the regression equation y = 64.958x-0.0191 obtained from the standard tannic acid calibration curve. A separate solution containing 0.8 mL of Folin-Ciocalteau reagent and 2 mL of aqueous sodium carbonate (75 g/L) with the volume adjusted to 10 mL mark using distilled water was used as the blank solution. Determination were performed in triplicate. Total phenolic content (TPC) in the *H. madagascariensis* fresh flowers as tannic acid equivalents (TAE) was calculated using the formula: TPC= (C x DF)/w´ where TPC is the total phenolic content in mg TAE/g of the fresh flowers, C is the concentration (mg/mL) of tannic acid calculated from the regression equation, DF is the test sample dilution factor (1000), w is the weight (g) of the *H. madagascariensis* fresh flowers that was extracted.

Determination of the total flavonoid content

This was determined by the $AlCl_3$ colorimetric method [21] with modification as earlier reported [15] and the total flavonoid content (TFC) expressed as milligram quercetin equivalent per gram of fresh flowers. Briefly, reference quercetin stock solution (0.5 mg/mL) was prepared in methanol. Using a micropipette, aliquots: 0.10, 0.20, 0.40, 0.60, 0.8 and 1.0 mL of this stock solution were separately transferred into their respective 10 ml volumetric flask labeled Q1-Q6. To the content of each of these volumetric flasks Q1-Q6 was separately added 0.5 mL of 10% $AlCl_3$ and the volume adjusted to 10 mL mark with methanol. This was thereafter left to incubate in the dark for 30 minutes for complete reaction. The absorbance of each of the reaction mixtures in Q1-Q6 was taken at 430 nm and the calibration curve obtained from a plot of absorbance against concentration using Microsoft Excel 2013 package and the regression equation noted. For the *H. madagascariensis* fresh flowers, 1.004 g of the fresh flowers was exhaustively extracted with methanol and the volume of the methanol filtrate adjusted to 50 ml in a 50 ml volumetric flask. A 0.5 ml aliquot of this resulting solution was then transferred to a 10 mL volumetric flask followed by the addition of 0.5 mL of 10% $AlCl_3$ and the volume adjusted to 10 mL mark with methanol. This was thereafter left to incubate in the dark for 30 minutes for complete reaction. The absorbance of the reaction solution was then taken at 430 nm and the total flavonoid content TFC calculated from the regression equation y = 63.7x-0.0495 obtained from the standard quercetin calibration curve. Methanol was used as the blank solution. Determination were performed in triplicate. Total flavonoid content (TFC) in the *H. madagascariensis* fresh flowers as quercetin equivalents (QE) was calculated using the formula: TFC= (C x DF)/w´ where TFC is the total flavonoid content in mg QE/g of the fresh flowers, C is the concentration (mg/mL) of quercetin calculated from the regression equation, DF is the test sample dilution factor (1000), w is the weight (g) of the *H. madagascariensis* fresh flowers that was extracted.

Results and discussion

The yield of the extract and fractions were: FLE (12.96% w/w), FLA (3.10% w/w) and FLD (6.10% w/w). As stated in Table 1, triterpenoids, cardiac glycosides, carbohydrate derivatives and phenolics like flavonoids are the secondary plant metabolites present in the flowers with alkaloids, anthraquinones, saponins and cyanogenic glycosides absent. From the results of the evaluation of the antioxidant properties (see Table 3), a total phenolic content (TPC) of 23.01 mg tannic acid equivalent per g fresh flower, and total flavonoids content (TFC) of 2.90 mg quercetin equivalent/g fresh flower were gotten. TPC and TFC are quantitative phytochemical data as well as used to predict antioxidant potentials of plants. The TPC and TFC results are in agreement with the observed presence of phenolics (see Table 1) from the qualitative phytochemical sxreening of the flowers of *H. Madagascariensis*. The total phenolic content assay is a quantitative estimation of the crude content of phytophenolics. It gives an aggregation of the content of all classes of phytophenolic compounds that include: the simple phenols and phenolics acids, phenyl propanoids, coumarine, tannins and flavonoids among others. The total flavonoids content assay is however specific for only the flavonoids class of phytophenolic compounds. This explains the lower TFC value obtained compared to the TPC (see Table 3). This trend is in agreement with earlier report for the fresh fruits (another morphological part) of this plant [15]. The result of the preliminary FRS assay (Table 2) showed that the crude ethanol extract (FLE) and its non-polar dichloromethane (FLD) and polar aqueous (FLA) fractions of *H. madagascariensis* flowers exhibited FRS activity with the polar FLA fraction having the significantly ($p< 0.05$) highest FRS activity. The observed FRS activity for the test extract and fractions were found also to be concentration dependent (see Figure 1) with a trend in half maximal inhibitory concentration IC_{50} (µg/ml)) of: FLD (16.80) >Ascorbic acid (12.17) >FLE (11.36) > FLA (7.36) calculated from the regression curve (see Figure 1). This further showed that the FLA has the highest FRS activity. The IC_{50} is a measure of how effective a substance is able to inhibit a specific biological function. The lower the IC_{50} value the more potent the inhibitory action of the test substance. Phytophenolic compounds which includes flavonoids, are known to have good antioxidant properties [22-25] The presence flavonoids and related phytophenolics in the *H. madagascariensis* fresh flowers could offers a plausible rationale for the promising FRS activity. Clinically oxidative stress is closely associated with the complications seen in several diseases treated with *Harungana madagascariensis* in folklore such as: anaemia [26-27], diabetes [28] bacterial infections [29-30], diarrhoea and gastro-intestinal disorders [31-32], and malaria [27]. Uncontrolled generation of radicals during human physiology is a leading predisposing cause of oxidative stress. Antioxidants substances help in the alleviation of oxidative stress. This they do by quenching the action of radicals when they donate an electron to the radical [33-34]. Hence they are vital in the maintenance of health, well-being, and in the prevention and management of oxidative stress and ageing process.

Table 1: Phytochemical screening result for the flowers of *H. madagascariensis*

Screened phytochemicals	Flowers
Anthraquinones: (Borntrager's test for free and combined)	-
Triterpenoids: Liebermann-Burchard	+
Salkowski test	+
Cardiac glycosides: Kedde (Cardenolide) test	+
Keller-Killiani (Deoxysugar) test	+
Saponins: (Frothing test)	-
Carbohydrate: Molisch test	+
Fehlings test	+
Cyanogenic glycosides: (alkaline picrate paper test	-
Alkaloids: (Dragendorf test)	-
Phenolics: $FeCl_3$ test	+
Phlobatannins test	-
Shinoda(Flavonoids) test	+

Key: present (+), absent (-)

Table 2: Preliminary free radical scavenging activity for the crude extract and fractions of *H. madagascariensis* flowers at 100 µg/ml

Test extracts/fractions	% Inhibition of DPPH activity
FLE	93.67±2.80
FLD	81.11±0.00
FLA	95.31±0.00

Key: the flower crude ethanol extract (FLE), flower dichloromethane fraction (FLD), flower aqueous fraction (FLA). Values are mean ± standard deviation, n=2

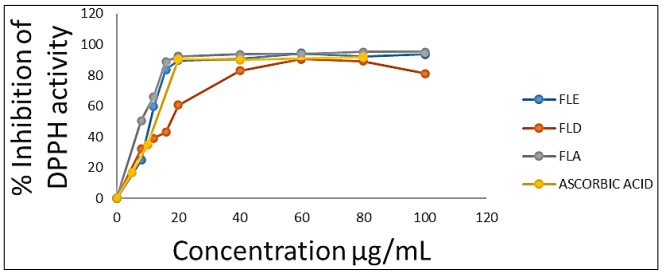

Fig 1: Concentration -response curve for the extract and fractions of H. madagascariensis flowers and the reference antioxidant agent ascorbic acid

Key to Figure 1: The flower crude ethanol extract (FLE), flower dichloromethane fraction (FLD), flower aqueous fraction (FLA)

Table 3: Some Antioxidant indices of *Harungana madagascariensis* fresh fruits.

Antioxidant indices	Potency
Total phenolic Content**	23.01± 0.24 mg TAE/g fresh flower
Total flavonoid content**	2.90±0.06 mg QE/g fresh flower
FRS activity for FLE(IC_{50})	11.36 µg/mL*
FRS activity for FLD(IC_{50})	16.80 µg/mL*
FRS activity for FLA(IC_{50})	7.36 µg/mL*
FRS activity for Ascorbic acid (IC_{50})	12.17 µg/mL*

Key: the flower crude ethanol extract (FLE), flower dichloromethane fraction (FLD), flower aqueous fraction (FLA) *extrapolated from regression plot in Figure 1. **Values are mean ± standard deviation, n=3

Conclusion

This study which is the first time report on the antioxidant and phytochemical properties of the flowers of *H. madagascariensis* a plant widely used in ethno-medicine showed that triterpenoids, cardiac glycosides, phytophenolics like flavonoids, and carbohydrate derivatives are present in the flowers while anthraquinones, alkaloids, saponins and cyanogenic glycosides are however absent. The observed trend in free radical scavenging activity which could be correlated to the presence of the phytochemicals especially the phytophenolics, offers an opportunity for the flower of this plant to be exploited as a veritable source of antioxidants for drug development.

Conflict of interest

There is no conflict of interest as regards the publication of this manuscript.

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