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Evaluation of the *in vitro* and *in vivo* antioxidant activity of aqueous and hydroethanolic extracts of *Harungana madagascariensis* trunk scores

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Abstract
Harungana madagascariensis (Hypericaceae) is a plant traditionally used by the people of Côte d'Ivoire and West Africa to treat malaria and gastrointestinal complications. With a view to enhancing the value of Ivorian plants, the antioxidant activity of aqueous and hydroethanolic extracts of the plant's stem bark were assessed *in vitro* and *in vivo*. The polyphenols in the various extracts were quantified spectrophotometrically, and the *in vitro* and *in vivo* antioxidant activity was assessed using DPPH, FRAP, TBARS and total antioxidant power tests. Quantification of the polyphenols showed that the hydroethanol extract was richer (429.6±17.11 mg EAG/g) than the aqueous extract (307.7±31.83 mg EAG/g). With regard to antioxidant activity *in vivo*, the hydroethanol extract had the best activity than vitamin C. However, the aqueous extract showed the best antioxidant activity *in vivo* and was close to that of ascorbic acid.

Keywords: *Harungana madagascariensis*, TBARS, total antioxidant capacity, *in vivo*

Introduction
Oxidative stress is defined as either excessive formation or incomplete elimination of reactive molecules such as reactive oxygen species (ROS) [1, 2]. ROS are implicated in many diseases (diabetes, atherosclerosis, Alzheimer's, cancer, arthritis, neurodegenerative diseases and the ageing process) [3, 4].
Antioxidants are used to combat these harmful species [5, 6]. These compounds are all molecules capable of directly inhibiting the production, limiting the propagation or destroying reactive oxygen species [7]. There are several types of antioxidant. Enzymatic antioxidants include superoxide dismutase, catalase and glutathione peroxidase, while non-enzymatic antioxidants include vitamin C, selenium, vitamin E, polyphenols and carotenoids [8, 9].
Protective mechanisms are often disrupted by various phenomena, and antioxidant supplements are essential to combat these harmful species [10]. Various forms of antioxidant products (butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylated hydroxyquinone) are used to protect against oxidative stress, but they are expensive [11]. These synthetic antioxidants also have side effects and are thought to be responsible for liver damage and carcinogenesis [12].
The use of plants as a natural remedy, as opposed to medicines sold in pharmacies, is enjoying a resurgence of interest among the population [13, 14]. Accessibility and availability are boosting the popularity of plants, as illustrated by the fact that 80% of Africans use plants to deal with their health problems [15, 16].
This preference is also linked to the richness of the flora in medicinal plants. *Harungana madagascariensis* (Hypericaceae), a species found in the flora of Côte d'Ivoire, came to our attention because of its traditional use to treat a variety of illnesses. In Côte d'Ivoire, the stem bark is used to treat hepatitis, malaria, river blindness, asthma, dysmenorrhoea, ulcers, toothache, nephrosis and gastrointestinal complications [17].
Studies carried out by [18] have demonstrated the *in vitro* antioxidant activity of stem bark extracts. A study of the antioxidant activity of Ivorian medicinal plants has been initiated with the aim of enhancing their value. The *in vitro* and *in vivo* antioxidant activity of the aqueous and hydroethanol extracts of the bark of the stem of *Harungana madagascariensis* were evaluated.

Materials and Methods

Plant material: The plant material used for this study was the trunk bark of *Harungana madagascariensis*. They were collected in the Abidjan (south of Côte d'Ivoire) and identified at the Centre National Floristique de Côte d'Ivoire, where a sample is kept (UCJ008607). The identified plant material (bark) was washed and dried at room temperature for 3 weeks in the laboratory. It was then pulverised and subjected to various extraction procedures.

Animal material: Male and female Wistar albino rats (*Rattus Norvegicus*) were used for the *in vivo* study. These animals were supplied by the vivarium of the Ecole Normale Supérieure (ENS) in Abidjan (Côte d'Ivoire). The rats were maintained under favourable rearing conditions. The rats were regularly fed with pellets and acclimatised in the animal house of the UFR des Sciences Pharmaceutiques et Biologiques of the Université Félix Houphouët-Boigny at a temperature of 25 °C for five (5) weeks. To weigh between 120 g and 140 g.

Preparation of the various extracts: To prepare the aqueous extract, one hundred grams (100g) of *H. madagascariensis* powder was added to one litre of distilled water and then macerated. The homogenate was then successively filtered twice on cotton and subsequently on Whatman 3 mm filter paper. The filtrate obtained was oven dried at 50 °C [19].
The method described by [19] was used to prepare the 70% hydroethanolic extract of *H. madagascariensis*. To do this, one litre of the hydroethanolic solution (ethanol/water 70:30) was added to one hundred grams (100g) of *H. madagascariensis* powder. The mixture was homogenised using a magnetic stirrer for 24 hours. The homogenate was then successively filtered twice on cotton wool and subsequently on Whatman 3 mm filter paper The filtrate obtained was dried in an oven at 50 °C.

Determination of total polyphenols: The method described by [20] was used to determine the total polyphenols. A volume of 2.5 mL of Folin-Ciocalteu reagent diluted 1:10 was added to 30 µL of extract. The mixture was incubated in the dark at room temperature for 2 minutes, and 2 mL of calcium carbonate solution (with the concentration of 75 $g.L^{-1}$) was added. The mixture was then kept in a water bath at 50 °C for about 15 minutes and rapidly cooled. The absorbance was read at 760 nm and distilled water was used as a blank. Calibration lines were built using gallic acid at different concentrations as a reference. The concentration of polyphenols was expressed in grams per litre of extract equivalent in gallic acid (g.L-1, Eq AG).

Study of antioxidant activity

Measurement of antioxidant activity *in vitro*

DPPH (2,2'-diphenyl-1-picrylhydrazyl) test: The anti-free radical activity of aqueous (EAqHM) and ethanolic extracts of *H. madagascariensis* (EEtHM) was measured by the DPPH test according to the method described by [21]. A series of concentrations (0-200 µg/ml) of extract and vitamin C were prepared in a 70% ethanol/water solution. A volume of 100 µL of this solution was then added to 3.9 mL of DPPH (70 µM) prepared in methanol. After homogenisation, the mixture was incubated at room temperature (25 °C) in the dark. After 15 minutes incubation, the absorbance was read at 517 nm against a blank containing methanol only. The percentage inhibition (PI) of the DPPH radical was calculated according to the following equation.

$$PI = \frac{A_0 - A_{extract}}{A_0} \times 100$$

A_0 = absorbance (control)
A extract = absorbance (extract)

The $IC_{50,}$ which is the concentration of plant extract or vitamin C responsible for 50% inhibition of DPPH radicals, is determined from the graph showing the percentage of DPPH inhibition as a function of extract or vitamin C concentrations.

FRAP test: It was performed according to the method described by [22]. A volume of 3500 µL FRAP reagent was added to 140 µL extract dissolved in methanolic solution. After 30 min incubation in the dark, the absorbance was read at 593 nm. Trolox was used as a positive control.

Study of antioxidant activity *in vivo*

Induction of oxidative stress by carrageenan: Oxidative stress was induced by injection of carrageenan into the right hind leg of the rats. Prior to the experiment, the rats, which weighed 130 grams, were fasted for 16 hours, then divided into 9 batches of 6 rats and orally pre-treated with the following substances.

- **Batch 1:** Receiving a 9% NaCl + Caragenin solution.
- **Batch 2 (White control):** Receiving only a 9% NaCl solution.
- **Batch 3, 4 and 5:** Treated, receiving a solution of EAqHM extract at 150, 300 and 600 mg/kg CP.
- **Batch 6, 7 and 8:** Treated, receiving an EEtHM extract solution of 150, 300 and 600mg/kg CP.
- **Batch 9:** Reference control, receiving a Vitamin C solution. With the exception of batch 2, the animals in the various batches received 0.2 mL of 1% carrageenan suspension 1 h after treatment in the sub plantar zone of the left hind leg. Blood was taken from the rats 5 h later in heparinised tubes. The blood was then centrifuged and the serum used for the assays.

Determination of substances that react with thiobarbituric acid: The thiobarbituric acid reactive substances (TBARS) assay was performed according to the method described by [23]. For the preparation of serum proteins, 2.5 mL 20% (w/v) trichloroacetic acid (TCA) was added to 0.5 mL rat serum and the mixture was centrifuged at 1500 g for 10 min. Next, 2.5 mL of sulphuric acid and 2 mL of 0.2% thiobaturic acid (MDA) were added to the sediment. The reaction mixture was stirred and incubated for 30 min in a water bath. After the addition of 4 mL n-butanol, the solution was centrifuged, cooled and the absorbance of the supernatant was read at a wavelength of 352 nm. The calibration curve was plotted using different concentrations of 1,1,3,3-tetramethoxypropane (0.0;1.875;2.380;4.760;9.530;19.050;38.110) as standard to determine the concentration of TBA-MDA adducts in each sample.

Assessment of total antioxidant power: The FRAP test was performed according to the method described by [22]. It measures the reducing power of antioxidants present in a mixture by their ability to reduce ferric tripyridyltriazine (Fe3+ -TPTZ) to ferrous ion (Fe2+ -TPTZ) at an acidic pH.

The FRAP reagent consisted of 300 mmol. L-1 acetate buffer, pH = 3.6;10 mmol.L-1 TPTZ in 40 mmol.L-1 HCl and 20 mmol.L-1 FeCl3 6 H2 O in a ratio of 10:1:1. A volume of 900 µL FRAP reagent, previously incubated at 37°C, was added to 70 µL distilled water and 30 µL sample. After incubation at 37 °C for 30 minutes, the absorbance was read at 593 nm. A concentration ranges from 0 to 2000 µM of ferrous sulphate (FeSO4, 7 H2 O) reducing standard was prepared and used to determine the FRAP values of the extracts and the standard antioxidant (vitamin C).

Statistical analysis: The results were expressed as means plus or minus standard deviation. Graphical representation of the data was carried out using Graph Pad Prism 5.0 software. Statistical analysis of the different results was carried out using analysis of variance (One Way Anova) followed by Dunnett's multiple comparison test. *** ** $p \leq 0.01$, ≤ 0.01 highly significant difference ** $p \leq 0.01 \leq 0.01$ highly significant difference * $0.01 < p \leq 0.05$: significant difference and $p > 0.05$, non-significant difference.

Results and Discussion

Quantity of polyphenols

Figure 1 showed the total polyphenol contents in the EAqHM and EEtHM extracts. The total polyphenol content of the EEtHM extract (429.6±17.11 mg EAG/g) was significantly higher than that of the EAqHM extract (307.7±31.83 mg EAG/g).

***In vitro* antioxidant activity of extracts**

DPPH test: The results of the free radical activity of vitamin C and the EAqHM and EEtHM extracts on the DPPH free radical are shown in Figure 2. The IC50 values were 10.33±0.08 µg/mL for vitamin C, 12.28±0.07 µg/mL for EAqHM extract and 6.72±0.08 µg/mL for EEtHM extract (Table I). The EAqHM extract was less active than vitamin C, whereas the EEtHM extract was more active than vitamin C.

FRAP test: Estimation of the total antioxidant capacity of the extracts showed that the EEtHM extract had a higher antioxidant capacity (456.8±16.92 µmol Eq Trolox/g EXS) than the EAqHM extract (266.0±4.69 µmol Eq Trolox/g EXS) (Table II).

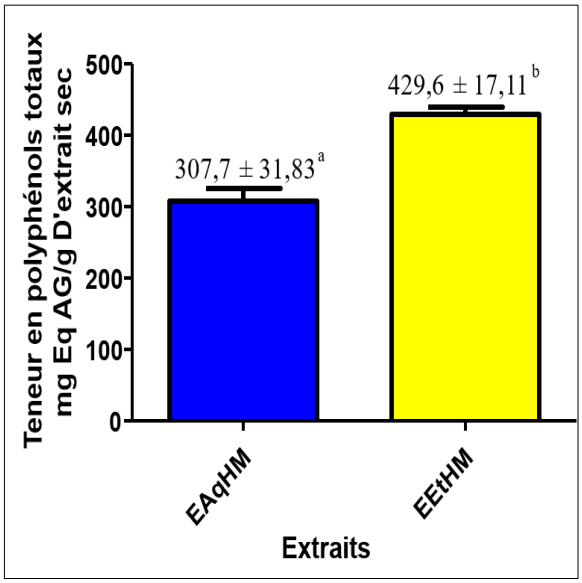

Fig 1: Total polyphenol content of EAqHM and EEtHM extracts

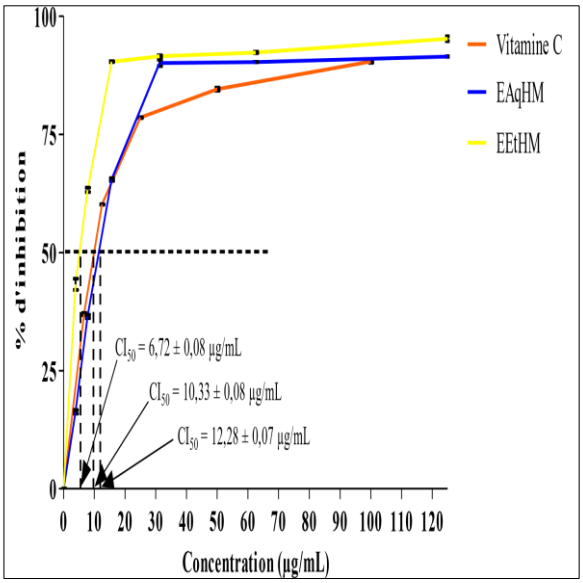

Fig 2: Percentage change in DPPH radical inhibition

Table 1: IC_{50} of vitamin C and EAqHM and EEtHM extracts for the DPPH test

	Vitamin C	EAqHM	EEtHM
IC_{50} (µg/mL)	10.33±0,08[a]	12.28±0.07[b]	6.72±0.08[c]

Means for vitamin C and extracts with superscripts a, b and c are significantly different from the smallest to the largest mean at p≤0.05.

Table 2: Antioxidant capacity values for EAqHM and EEtHM extracts

FRAP (µmol Eq Trolox/ g EXS)
EAqHM 266.0±4.69 [b]
EEtHM 456.8±16.92 [a]

Means for vitamin C and extracts with superscripts a and b are significantly different from the smallest to the largest mean at p≤0.05.

***In vivo* antioxidant activity**

Lipid peroxidation by measuring substances that react with thiobarbituric acid (TBARS): The effect of vitamin C and EAqHM and EEtHM extracts on the concentration of TBAMDA (thiobarbituric acid-malondialdehyde) adduct is shown in Figure 3.

Carrageenan induced a significant ($p \leq 0.001$) increase in serum MDA levels in NaCl-pretreated rats compared to non-carrageenan intoxicated rats. A significant ($p \leq 0.001$) decrease in TBA-MDA adduct levels was observed when carrageenan-intoxicated rats were pre-treated with EAqHM (150 and 300 mg/kg bw) and EEtHM (300 and 600 mg/kg bw) extracts. The inhibition of lipid peroxidation by EAqHM and EEtHM extracts was similar to that of vitamin C.

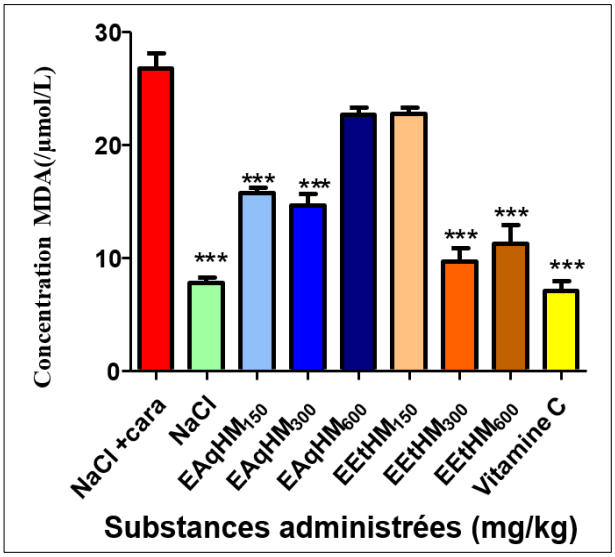

Fig 3: Inhibition of lipid peroxidation by vitamin C and EAqHM and EEtHM extracts

Total antioxidant power

The total antioxidant power of vitamin C and the EAqHM and EEtHM extracts is shown in Figure 4. Injection of carrageenan into NaCl pre-administered (untreated) rats induced a considerable decrease in Fe^{2+} ion concentration (resulting from the reduction of Fe^{3+}) compared to non-intoxicated control rats. However, animals intoxicated with carrageenan and pre-treated with different doses of EAqHM and EEtHM extracts and vitamin C had significantly higher Fe^{2+} concentrations ($p \leq 0.001$) than untreated intoxicated control rats.

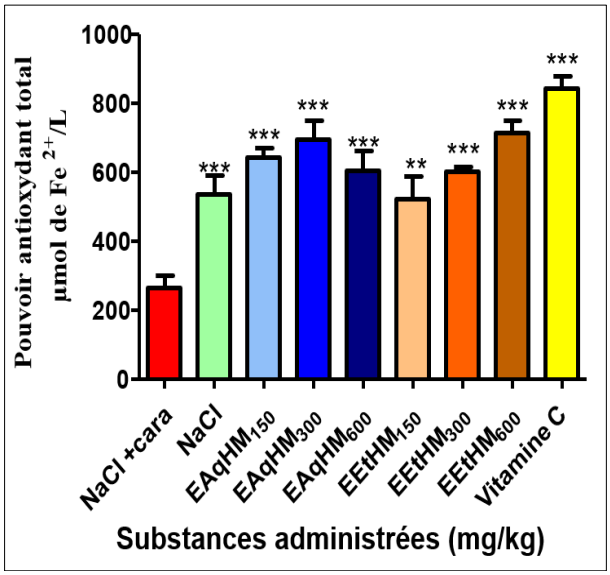

Fig 4: Total antioxidant power (*in vivo*) of vitamin C and EAqHM and EEtHM extracts

Discussion

This study is part of an effort to enhance the value of traditional medicine, more specifically in the search for antioxidant substances with the aim of producing Traditionally Improved Medicines (TIMs). This work focused on the aqueous and hydroethanolic extracts of the bark of the trunk of *Harungana madagascariensis*, which is part of the rich floral heritage of Côte d'Ivoire. The antioxidant activity has aroused interest because of the important role played by antioxidant compounds present in plants in the treatment and prevention of diseases caused by oxidative stress [24, 25].

As polyphenols are thought to be responsible for the antioxidant activity of plant sources [26], their levels in the different extracts were determined. These assays showed that the hydroethanol extract (429.6±17.11 mg EAG/g) was richer in polyphenols than the aqueous extract (307.7±31.83 mg EAG/g).

In fact, the solubility of polyphenols depends on the type of solvent used, their degree of polymerisation and their interaction with other components. For optimal extraction of polyphenols, ethanol is the most suitable solvent [27]. A study by [28] reported that ethanolic extracts had the highest total polyphenol content. Our results are higher than those obtained by [18] which were 134.24±3.71 mg GAE/g for the aqueous extract. Certain environmental conditions such as soil composition, temperature, precipitation and exposure to sunlight could explain this difference in content [29].

Concerning *in vitro* anti-free radical activity, the results showed that the hydroethanol extract had better reducing activity than the aqueous extract, irrespective of the test used. These chemical tests are based solely on chemical reaction mechanisms. In some cases, FRAP involves reduction while DPPH involves electron transfer [30, 31]. This result is closely related to the content of phenolic compounds in these different extracts. Some extract rich in phenolic compounds has a better antioxidant activity. These results are in agreement with those obtained by [32, 33] on the influence of the experimental conditions during the extraction of polyphenols. The EEtHM extract with an IC_{50} = 6.72±0.08 µg/mL showed a higher antioxidant activity than vitamin C (IC_{50} = 10.33±0.08 µg/mL), which is higher than the antioxidant activity of the EAqHM extract (IC_{50} = 12.28±0.07 µg/mL). Thus, EEtHM extract could effectively combat oxidative stress in the same way as vitamin C. Furthermore, our extracts (EAqHM and EEtHM) showed better antioxidant activities compared to some previous studies. Indeed, the ICs50 obtained in these different studies were 25 µg/mL, 63.63±2.42 µg/mL, 87.66±0.97 µg/mL and 147 µg/mL for methanolic extracts of the stem bark of H. madagascariensis [34-37]. However, our results are similar to those of [38] with the EEtHM extract (CI50 = 5.33 µg/mL). Since a substance that is very active *in vitro* may lose this antioxidant activity once in the body due to changes in certain physicochemical parameters, it was important to carry out an *in vivo* study to obtain an overall view of the antioxidant activity of the extracts tested.

TBARS and FRAP tests were performed to determine the *in vivo* antioxidant activity of EAqHM and EEtHM extracts of Harungana madagascariensis. In the TBARS test, a significant decrease in TBA-MDA adduct concentrations was observed when carrageenan-intoxicated rats were pre-treated with EAqHM and EEtHM extracts. Furthermore, using the FRAP test, carrageenan poisoned animals pre-treated with different doses of EAqHM and EEtHM extracts showed significantly higher Fe2+ concentrations than untreated poisoned control rats.

Our extracts therefore have good antioxidant activities *in vivo*, comparable to vitamin C.

The results of our study are similar to those of [39] who showed that the aqueous and hydroethanolic extracts of Trema guineesis leaves at doses of 100 and 200 mg/kg CP had a significant antioxidant effect.

This reducing capacity of the extracts is justified by their polyphenol content. According to [40], there is a relationship

between the content of phenolic compounds and the reducing power. The results of these *in vivo* tests confirm those obtained in the *in vitro* tests of the present study.

Conclusion

The discovery of natural resources from the plant kingdom is very important for the development of new therapeutic remedies. The present study focused on the species *H. madagascariensis*, an important plant in the Ivorian medicinal flora and widely used by traditional therapists. The study showed that aqueous and hydroethanolic extracts of the bark stem had better antioxidant activity *in vivo*. These extracts would be better candidates for the formulation of MTAs to overcome oxidative stress.

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