

**International Journal of Biochemistry Research
& Review**

7(4): 182-191, 2015, Article no.IJBcRR.2015.068
ISSN: 2231-086X

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***In vitro* and *In vivo* Antioxidant Activity of the Total
Dichloromethane-ethanol Extract of *Morinda
morindoides* (Baker) Milne-redh. (ETDE) (Rubiaceae)**

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Authors' contributions

This work was done as a team with all authors. Authors BGL and BC designed the study, performed the different tests and wrote this article. Author KMA performed the statistical analysis and managed literature. Authors NJD and DAJ checked results and managed the scientific research of the study. All authors read and approved the manuscript.

Article Information

DOI: 10.9734/IJBcRR/2015/17382

Editor(s):

(1) Richard A. Manderville, Departments of Chemistry and Toxicology University of Guelph, Canada.

Reviewers:

(1) Tra Bi Irie Otis, Biosciences Department, Cocody University, Ivory Coast.

(2) Anonymous, Cuba.

Complete Peer review History: <http://www.sciencedomain.org/review-history.php?iid=1039&id=3&aid=9546>

Original Research Article

**Received 11th March 2015
Accepted 28th April 2015
Published 2nd June 2015**

ABSTRACT

Purpose: our work consisted in studying the antioxidant activity of the dichloromethane-ethanol total extract (Baker) milne-redh., a Rubiaceae with antihypertensive activity.

Methodology: The antioxidant activity was determined *in vitro* by the test of 2,2'-diphenyl-1-picrylhydrazyl (DPPH), the test of inhibition of lipid peroxidation by TBARS method and *in vivo* by determining activities of SOD, CAT and the concentration of NO in the organs of rats made hypertensive by adrenalin (ADR). *In vivo*, Rats were divided into 7 lots of 5 rats each. The control lot received daily orally distilled water for 14 days. The other lots received adrenalin (ADR) at dose

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of 1 mL/kg by intraperitoneal injection for 8 days. After installation of hypertension, lot MNT (Lot sick untreated or positive control) has not undergone any treatment. Rats of lots ETDE 500 and ETDE 1500, received orally ETDE at doses of 500 and 1500 mg/kg bw for 6 days. Rats of lots TEN (tenordate) 10 and TEN 20 received orally the tenordate at doses of 10 and 20 mg/kg pc for 6 days. After the 6 days of treatment, the rats were decapitated and their hearts, kidneys, livers and aortas were collected for the determination of oxidative stress parameters.

Results: Our results showed that ETDE, *in vitro*, reduces greatly the DPPH with a IC_{50} of $21.45 \pm 1.53\%$ and inhibits $49.66 \pm 3.83\%$ of lipid peroxidation. *In vivo*, ETDE reduced significantly and normalizes the activity of SOD, CAT and normalizes the concentration of NO of rats made hypertensive.

Conclusion: ETDE by its antioxidant action on free radicals may be beneficial in the treatment of high blood pressure.

Keywords: Antioxydative activity; oxidative stress; *Morinda morindoides*.

1. INTRODUCTION

Cardiovascular diseases are responsible for about 17 million deaths per year in the world, nearly a third of all deaths [1]. On this number, the complications of high blood pressure are responsible for 9.4 million deaths per year [2]. Hypertension due to its prevalence has become a public health problem. But many studies have shown that increased oxidative stress may play an important role in high blood pressure (BP) and therefore in the occurrence of hypertension [3,4,5]. Oxidative stress is defined as an imbalance between the biochemical processes of production of ROS and those who are responsible for their control and elimination [6,7,8,9]. Oxidative stress can be the cause of the appearance of several often irreversible damage to the cells [10,11]. Clinical studies show an increase in the production of ROS in patients with essential hypertension, renovascular hypertension, malignant hypertension or pre-eclampsia [12,13,14]. It is therefore obvious that any substance that reduces oxidative stress could have a positive impact on the treatment of hypertension. Oxidative stress involves a complex set of parameters and cannot be identified by a single method, as elaborate as it [15]. In this study, we investigated the antioxidative effect of an ethanol-dichloromethane extract of *Morinda morindoides* (Baker) Milne-Redh. (ETDE) *in vitro* by the test of DPPH and the test of lipid peroxidation (TBARS method) and *in vivo* by the dosages of superoxide dismutase (SOD), catalase (CAT) and the concentration of nitric oxide (NO) in the organs of rats made hypertensive at ADR.

2. MATERIALS AND METHODS

2.1 Material

White albino male and female rats, Wistar strain aged 2 to 3 months are used for the study of oxidative stress of the extract. The rats were kept in plastic cages with stainless steel covers containing a bed of wood chips renewed every two days. The animals are fed regularly with rat pellets and received standard tap water as the drinking water in stainless steel cylinders.

The plant material is constituted by leaves of *Morinda morindoides* (Baker) Milne-Redh., collected to Gbahiri in the municipality of Lakota, South-western city of Côte d'Ivoire, between January and February 2012. These leaves were dried and powdered for the preparation of the extract.

2.2 Methods

2.2.1 Preparation of the extract

The dichloromethane-ethanol extract of *Morinda morindoides* (Baker) Milne-Redh. (ETDE) was prepared according to the method described by ZIRIHI et al. [16].

According to this method, one hundred and fifty grams (150 g) powder of *Morinda morindoides* (Baker) Milne-Redh were dissolved in eight hundred milliliters (800 mL) of a mixture of dichloromethane-ethanol solvent (1/3 (V / V)). The mixture is homogenized for 24 hours at laboratory temperature (25-30°C) using a magnetic stirrer IKAMAG RCT. The homogenate is filtered twice on cotton wool and once on Whatman 3 mm paper filter. The recovered

filtrate is evaporated using a Büchi rotary evaporator. We got a greenish paste that forms the total dichloromethane-ethanol extract of *Morinda morindoides* (Baker) Milne-redh. (ETDE). Different doses for testing were prepared from this extract.

2.2.2 In vitro evaluation of antioxidant activity of ETDE

2.2.2.1 Measurement of the antioxidant activity by the DPPH test

The measurement of the antioxidant activity of ETDE is performed by the test of 2,2-diphenyl-2-picrylhydrazyl (DPPH) according to the method of Parejo et al. [17]. This method is used to evaluate the ability of the ETDE to fix free radicals by measuring the decrease of the violet coloration due to the reduction of radicals produced by DPPH.

1 mg of ETDE is mixed with 10 mL of methanol to prepare the mother solution. Thus, from a mother solution ETDE (0.1 mg/mL), a range of concentrations is prepared by successive dilution twice. The same volume of a methanolic solution of DPPH was added to each concentration of the extract. After 30 min incubation at room temperature (25°C), protected from light, the absorbance is read in a spectrophotometer at 517 nm against the blank sample (medium consisting solely of methanol). Vitamin C (0.1 mg/mL), prepared under the same conditions is used as standard. Inhibition of DPPH radicals are calculated using the formula:

$$\text{DPPH inhibition (\%)} = (1 - \text{sample OD} / \text{OD control}) \times 100$$

Where OD control is the absorbance of the DPPH solution without plant extract and sample OD is the absorbance of the reaction medium containing DPPH and the plant extract or vitamin C. Concentration of ETDE causing 50% inhibition of DPPH radicals (IC₅₀) is determined on a curve showing the inhibition of DPPH radicals depending on the concentration of the ETDE then compared to that of vitamin C.

2.2.2.2 Measurement of inhibition of lipid peroxidation by the method of TBARS

The method of Choi et al. [18] using an induction of lipid peroxidation by ascorbic acid/iron sulfate (Fenton reaction) has been adapted for this test. Six hundred (600) µL of ETDE at a

concentration of 500 µg/mL, respectively, is mixed with 300 µL of Tris-HCl buffer solution (pH7.5; 20 mM), 500 µL of linoleic acid concentration of 20 mM and 100 µL of 4 mM concentration of iron sulfate. Peroxidation begins after addition of 100 µL of 5 mM concentration of ascorbic acid. Each reaction mixture obtained was incubated for 60 minutes at 37°C in a waterbath. After this step, 2 mL of TCA (10%) are added to all tubes. Then, 1 mL aliquot collected in each of the reaction mixtures prepared previously, are added 1mL of TBA (1%). The reaction mixtures obtained are placed in boiling water bath at 95°C for 20 minutes. Gallic acid and distilled water are respectively used as reference molecule and as a negative control. Absorbance is read in a spectrophotometer at 532 nm and the percent inhibition of linoleic acid is determined according to the following equation:

$$\text{Inhibition (\%)} = (1 - \text{sample OD} / \text{OD negative control}) \times 100$$

2.2.3 Evaluation In vivo of antioxidant activity of rats made hypertensive

ETDE is an extract that we tested on high blood pressure and doses used in this experiment are those that showed interesting antihypertensive activity.

Thirty five rats aged 2 to 3 months were used. These rats were divided into 7 groups of 5 rats each including a control lot and six test lots.

Rats of the control lot received orally distilled water according to their weight during 14 days and rats of test lots received adrenalin at 1 mL/kg during 8 days. After these 8 days of hypertension induction, organs of rats of TM lot were collected. Rats of MNT lot (Lot sick untreated or positive control) have not undergone any treatment during the six days of treatment of other tests lots. This is the lot that served as a control for lots made hypertensive and treated. Rats of ETDE 500, ETDE 1500 lots received by gavage respectively ETDE at doses of 500 and 1500 mg/kg bw then rats of TEN 10 and TEN 20 received by gavage tenordate at doses of 10 and 20 mg/kg bw during 6 days (treatment period).

After the 6 days of treatment, rats were sacrificed by decapitation, their organs (liver, heart, aorta and kidney) were collected and ground to the assay parameters markers of oxidative stress.

2.2.3.1 Determination of the activity of superoxide dismutase (SOD)

The SOD activity was assayed by testing the Nitro Blue tetrazonium (NBT). The nitro blue tetrazonium (NBT) is reduced by NADPH in the presence of superoxide anion (O₂⁻) and gives a dark violet chromophore. Whereas the SOD eliminates superoxide anion (O₂⁻), the intensity of the coloration of the chromophore is proportional to the activity of SOD in the medium. However, the SOD activity was measured using the following method. In a test tube were added 5 µl of the homogenate of the organ and 2 mL of the reaction mixture (sodium cyanide 2.10⁻⁵ M; NBT 1,76.10⁻⁴ M solution; ETDE 6,6.10⁻³ M; riboflavin 2.10⁻⁶ M; 10⁻² M methionine and 3 mg of NADPH), and the mixture was irradiated with a 15 Watt lamp for 10 minutes. The absorbance is then measured at 560 nm. The values of SOD activity are in UI.mg⁻¹ proteins.

2.2.3.2 Determination of activity of catalase (CAT)

The catalase activity was measured in the homogenate of the organ spoke spectrometric method. Indeed catalases are responsible for the degradation of H₂O₂ in H₂ and O₂. The assay consists of measuring the decrease in absorbance due to the disappearance of the oxygen peroxide which is the substrate of the enzyme. This reduction in the oxygen peroxide which is proportional to the activity of the catalase was determined by preparing in a quartz measurement cell, a substrate solution consisted of 1 mL of phosphate buffer (KH₂PO₄, 0.1 M, pH 7.4), 0.950 mL of H₂O₂ (0,019M), 0.025 mL of the enzyme source (fraction aorta, kidney, liver and heart). The reaction was followed by recording the absorbance at 560 nm each minute for two minutes. Enzyme activity is expressed in mmol H₂O₂ decomposed /µg prot.

2.2.3.3 Determination of nitric oxide (NO) concentration

The dosage of nitric oxide (NO) has been made from the Griess reagent, the preparation was made to protect from light. Sulfanilamide (1%) and the naphthyléthylenediamide (0.1%) were diluted (v/v) phosphoric acid (2.5%). The standard solution used was a solution of sodium nitrite (NaNO₂) in 1 mM concentration. The sodium nitrite solution (NaNO₂, 1 mM) was diluted in half in a series of 13 test tubes. In

tube1 containing 100 µl of NaNO₂ were added 100 µl of reagent GRIESS. The whole was homogenized by vortexing and 100 µl were collected for the tube 2. The optical density was read at 570 nm after 10 minutes. In each of the remaining 12 tubes were initially introduced 100 µl of distilled water. Dilution of NaNO₂ was then made as follows: at the tube 2 contents were added 100 µl of the solution taken in the tube 1 the whole was homogenized by vortexing and 100 µl of this mixture were removed and added to the tube 3 and so on to tube 13. To the last tube (No. 13), 100 µl of the mixture were removed and discarded. The contents of tubes 2 and 13 were then added 100 µl of Griess reagent and the absorbance of each tube was read after 10 minutes at 570 nm.

This first series of tubes has allowed to establish a calibration curve. The evaluation of the amount of NO in various homogenates was made as follows: in five (5) test tubes was introduced in 100 µl of homogenate order organ and 100 µl of Griess reagent. After thorough mixing, the optical density of each tube, the intensity of the staining mixture is proportional to the NO concentration was determined by spectrophotometer (Spectronic Genesys 20, Thermo) at 570 nm after 10 minutes of rest.

2.3 Statistical Analysis

The graphical representation of the data was performed using the Graph Pad Prism 5.0 software (Microsoft, USA). The mean value is accompanied by the standard error of the mean (Mean±SEM). The difference between the two values is considered significant when P <0.001. Statistical analysis of results was performed using analysis of variance (ANOVA).

3. RESULTS

3.1 Effect of ETDE on Oxidative Stress *In vitro*

The results of the inhibition of free radicals DPPH by ETDE and vitamin C are represented by Figs. 1 and 2. The graphical determination of the concentration of ETDE and vitamin C causing 50% inhibition of free radical DPPH (IC₅₀) yielded values of 21.45±1.53 and 2.27±0.26 g/mL, respectively, for ETDE and vitamin C. These results show that ETDE has good anti-radical activity.

3.1.1 Test of lipid peroxidation (TBARS)

Fig. 3 shows the results of lipid inhibition by ETDE and vitamin C (Vit C). The results show that the ETDE inhibits $49.66 \pm 3.83\%$ of lipid peroxidation while Vit C is the reference molecule exerts $61.47 \pm 1.58\%$ of inhibition.

3.2 Effect of ETDE and Tenordate on Oxidative Stress *In vivo* among Rats Made Hypertensive

3.2.1 Effect of ETDE and tenordate on SOD activity in rats made hypertensive

The effect of ETDE on SOD activity in rats made hypertensive at ADR is represented by Figs. 4 and 5. The ADR induced a significant increase ($p < 0.01$) of SOD activity in the aorta of 33.75%, in the heart of 23.5%, in the liver of 7.8% and in

the kidney of 8.81% compared to its activity in control rats. ETDE at the dose of 1500 mg/kg bw reduced significantly the SOD activity of 23.53% in the aorta, from 20.91% in the heart, of 8.30% in the liver and 8.38% in the kidney compared to its activity in control rats organs.

The ETDE at a dose of 500 mg/kg bw resulted in a significant reduction in SOD activity of 19.79% in the aorta, from 16.55% in the heart, in the 5.54% liver and 6.07% in the kidney compared with normotensive control rats. At a dose of 1500 mg/kg bw, ETDE reduced the SOD activity of hypertensive rats to normal, but this action is more pronounced in the kidney where the activity is reduced below the normal activity of control rats. The ténordate at a dose of 20 mg/kg bw also reduced SOD activity in these organs in the same proportions as the ETDE.

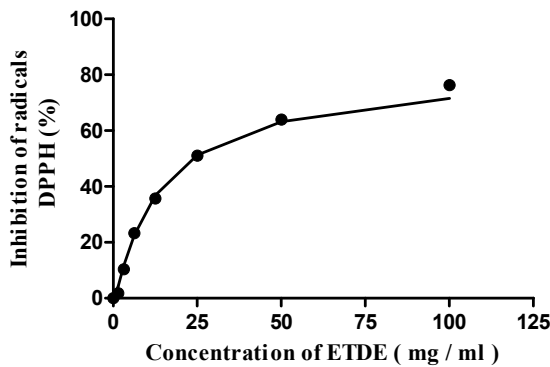


Fig. 1. Inhibition of DPPH in function of concentration of ETDE

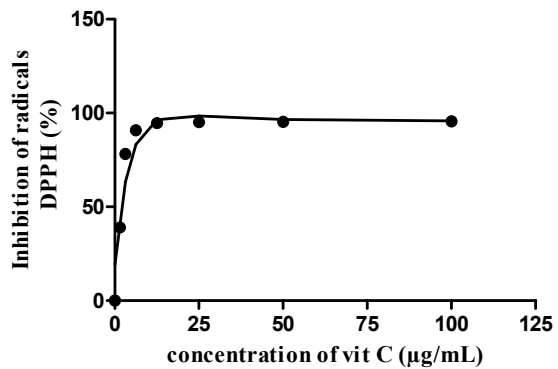


Fig. 2. Inhibition of DPPH in function of concentration of Vitamin C

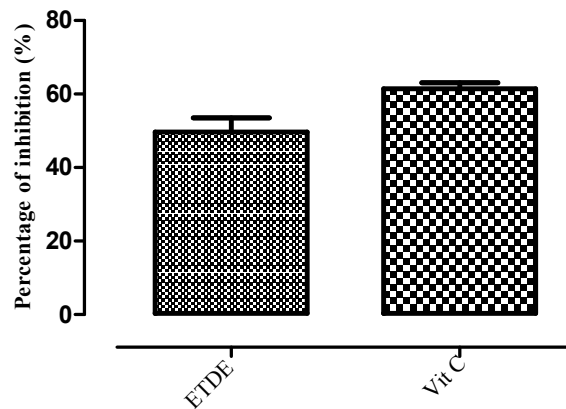


Fig. 3. Inhibition of lipid peroxidation by the TBARS method

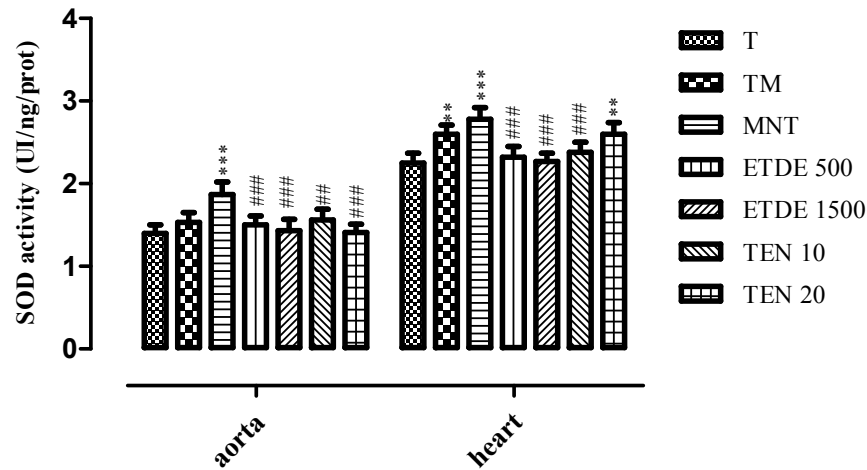


Figure 4: Effect of ETDE and tenordate on the SOD activity in the aorta and the heart of rats made hypertensive

Each bar represents the mean \pm SEM, $n = 5$. ** $P < 0.001$, *** $P < 0.001$; significant difference from the control group. ## $P < 0.001$, ### $P < 0.001$, significantly different from the untreated patient lot. T = control lot, TM = batch sick witness, MNT = sick untreated lot (positive control); ETDE 500 = hypertensive lot treated with ETDE at dose of 500 mg / kg bw; ETDE 1500 = hypertensive lot treated with ETDE at dose of 1500 mg/kg bw; TEN 10 lot = hypertensive lot treated with ténordate at dose of 10 mg/kg bw and TEN 20 = lot hypertensive lot treated with ténordate at a dose of 20 mg/kg bw

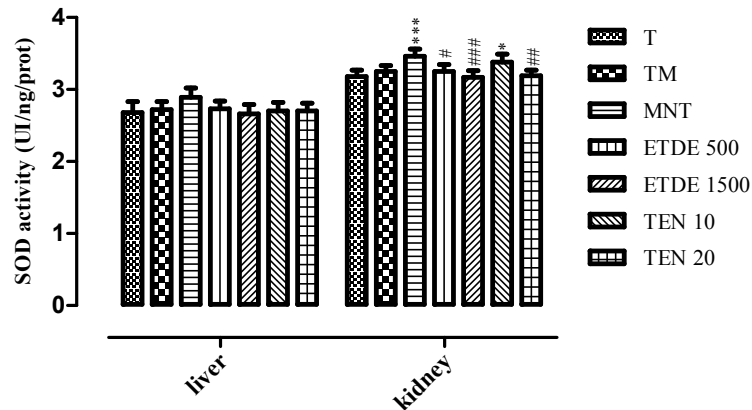


Figure 5: Effect of ETDE and tenordate on the SOD activity in the liver and the kidney of rats made hypertensive

Each bar represents the mean \pm SEM, $n = 5$. ** $P < 0.001$, *** $P < 0.001$; significant difference from the control group. ## $P < 0.001$, ### $P < 0.001$, significantly different from the untreated patient lot. T = control lot, TM = batch sick witness, MNT = sick untreated lot (positive control); ETDE 500 = hypertensive lot treated with ETDE at dose of 500 mg/kg bw; ETDE 1500 = hypertensive lot treated with ETDE at dose of 1500 mg/kg bw; TEN 10 lot = hypertensive lot treated with ténordate at dose of 10 mg/kg bw and TEN 20 = lot hypertensive lot treated with ténordate at a dose of 20 mg/kg bw

3.2.2 Effect of ETDE and tenordate on catalase activity in rats made hypertensive

Intraperitoneal injection of ADR to rats provoked a significant increase ($p < 0.001$) of the catalase activity of 40.23%, 56.29%, 79.02% and 87.41% respectively in aorta, heart, liver and kidney. The

ETDE at a dose of 1500 mg/kg bw caused a significant reduction ($P < 0.001$) of catalase activity in the aorta of 28.27%, in the heart of 32.25% in the 43.26% liver and kidney 49.81% compared to its activity in hypertensive rats. The tenordate at a dose of 20 mg/kg bw also reduced the CAT activity but it is still above the normal activity (Figs. 6 and 7).

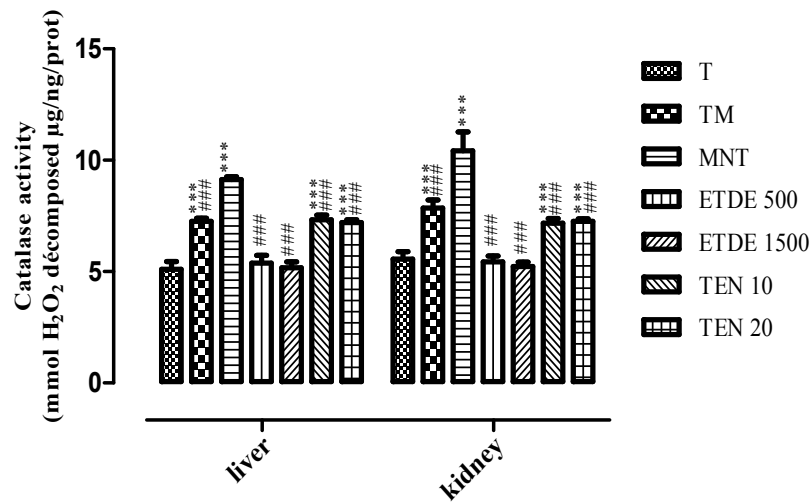


Figure 6: Effect of ETDE and tenordate on the catalase activity in the liver and the kidney of rats made hypertensive

Each bar represents the mean \pm SEM, n = 5. ** P < 0.001, *** P < 0.001; significant difference from the control group. ## P < 0.001, ### P < 0.001, significantly different from the untreated patient lot. T = control lot, TM = batch sick witness, MNT = sick untreated lot (positive control); ETDE 500 = hypertensive lot treated with ETDE at dose of 500 mg/kg bw; ETDE 1500 = hypertensive lot treated with ETDE at dose of 1500 mg/kg bw; TEN 10 lot = hypertensive lot treated with ténordate at dose of 10 mg/kg bw and TEN 20 = lot hypertensive lot treated with ténordate at a dose of 20 mg/kg bw

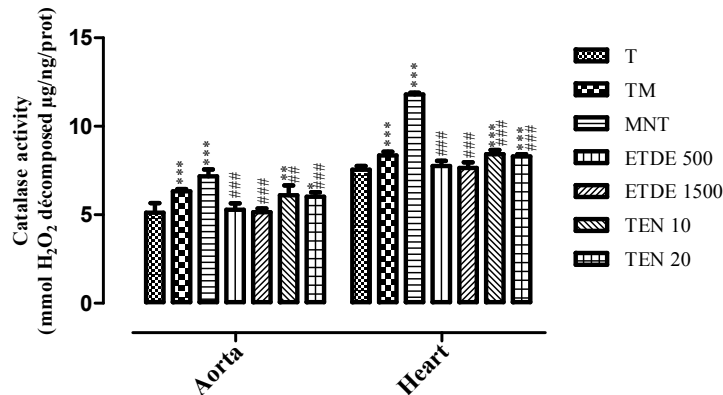


Figure 7: Effect of ETDE and tenordate on the catalase activity in the aorta and the heart of rats made hypertensive

Each bar represents the mean \pm SEM, n = 5. ** P < 0.001, *** P < 0.001; significant difference from the control group. ## P < 0.001, ### P < 0.001, significantly different from the untreated patient lot. T = control lot, TM = batch sick witness, MNT = sick untreated lot (positive control); ETDE 500 = hypertensive lot treated with ETDE at dose of 500 mg/kg bw; ETDE 1500 = hypertensive lot treated with ETDE at dose of 1500 mg/kg bw; TEN 10 lot = hypertensive lot treated with ténordate at dose of 10 mg/kg bw and TEN 20 = lot hypertensive lot treated with ténordate at a dose of 20 mg/kg bw

3.2.3 Effect of ETDE and ténordate on the concentration of nitrogen monoxide (NO) in the aorta of hypertensive rats

Fig. 8 shows that administration of ADR to rats resulted in a significant decrease in the concentration of NO in the aorta of 34.78% compared to normotensive control rats. The ETDE, administered to rats at doses of 500 and

1500 mg/kg bw increased significantly the concentration of NO in the aorta, respectively (p < 0.001) of 168.75% and 218.75% compared to the untreated patient lot and was normalized to the control lot. The tenordate at doses of 10 and 20 mg/kg bw had no significant effect (p < 0.001) on the concentration of NO compared to the untreated patient lot.

4. DISCUSSION

To assess the *in vitro* antioxidant activity of ETDE, we used two tests: the test of the reduction of DPPH radical and the inhibition assay of lipid peroxidation by the TBARS method. Indeed, given the complexity of the process of oxidation and variety of antioxidants, there is not a universal method to quantitatively measure, and in a precise way, the antioxidant activity of a substance. We must therefore combine responses of different complementary tests to have an indication of the antioxidant capacity of the test sample [19,20].

The test of the reduction of DPPH showed a strong antioxidant activity of ETDE with an IC_{50} of $21.45 \pm 1.53 \mu\text{g/mL}$ compared to the reference molecule Vitamin C which has an IC_{50} of $2.27 \mu\text{g/mL}$. The ETDE therefore contain one or several molecules able to transfer an electron to the radical DPPH and reducing the latter in DPPH-H. The results of the lipid peroxidation test showed a very good antioxidant activity of ETDE with a percentage of inhibition of $49.66 \pm 3.83\%$ compared to gallic acid with a percentage of inhibition of $61.47 \pm 1.58\%$. The lipid peroxidation which is the oxidation of the polyunsaturated fatty acids by the free radicals or the enzymes of the body, has for main targets cellular membranes and LDL (Low Density Lipoproteins) very rich in polyunsaturated fatty acids [21,22]. The

sensitivity of fatty acids to oxidation is correlated with their degree of unsaturation [21]. Indeed, lipid oxidation by free radicals generates lipid peroxides which are able to generate other free radicals which become very high in the body.

These free radicals will cause a decrease in the bioavailability of nitric oxide, a vasodilator, or increase in intracellular calcium which causes high blood pressure [23,24]. The inhibition of lipid peroxidation by ETDE is very beneficial in the fight against oxidative stress and thus for the treatment of hypertension. The determination of the specific activity of superoxide dismutase (SOD), an enzyme which catalyzes the dismutation of the superoxide anion (O_2^-) in water and hydrogen peroxide reveals a significant increase ($p < 0.001$) of this activity in rats made hypertensive compared with the control lot. The increase of SOD activity would be secondary to the production of superoxide anion [25]. Our results show that the SOD activity is more pronounced in the kidney, heart and aorta. The treatment of animals by ETDE reduced significantly the SOD activity to until the normalization in the kidney, heart and aorta of rats made hypertensive compared to the control lot. The ETDE would capturing the superoxide anion to eliminate it, the effect of antioxidants been to just get the superoxide anion [26]. The administration of the ADR to rats increased significantly ($p < 0.001$) the CAT activity in the

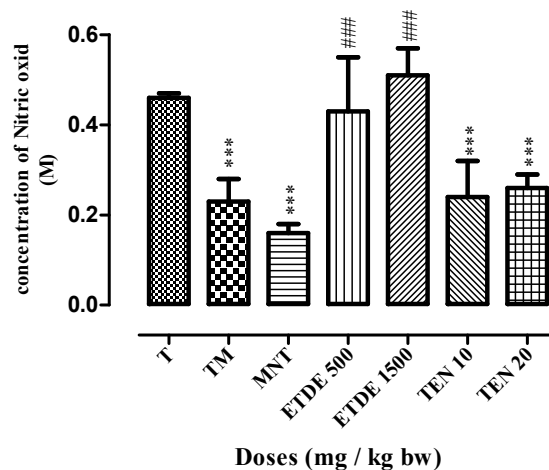


Figure 8: Effect of ETDE and tenordate on the nitric oxide concentration in the aorta of rats made hypertensive

Each bar represents the mean \pm SEM, $n = 5$. ** $P < 0.001$, *** $P < 0.001$; significant difference from the control group. ## $P < 0.001$, ### $P < 0.001$, significantly different from the untreated patient lot.
 T = control lot, TM = batch sick witness, MNT = sick untreated lot (positive control); ETDE 500 = hypertensive lot treated with ETDE at dose of 500 mg/kg bw; ETDE 1500 = hypertensive lot treated with ETDE at dose of 1500 mg/kg bw; TEN 10 lot = hypertensive lot treated with tenordate at dose of 10 mg/kg bw and TEN 20 = lot hypertensive lot treated with tenordate at a dose of 20 mg/kg bw

aorta, liver, heart and kidney compared to rats of the control lot. The increase in CAT activity is indicative of a high production of hydrogenperoxide (H₂O₂) in these organs. CAT, through its action, defuses the oxidative potential of H₂O₂ by transforming it into H₂O and O₂ [27]. The ETDE administered to hypertensive rats reduced in significant way (p <0.001) the CAT activity compared to the sick control lot and normalized it compared to the control lot. This reduction in CAT activity suggests that ETDE has reduced the amount of H₂O₂ or its production in these organs. It should be noted that in the liver, SOD had no significant effect on the oxidative stress then on the superoxide anion thus suggesting there was no generation of H₂O₂ by reaction of SOD. H₂O₂ reduced here by the CAT would be produced by other enzymes such as oxidases present in peroxisomes [28]. The ténordate, a reference antihypertensive, had no significant effect on CAT activity of rats made hypertensive.

ADR caused a significant reduction in the concentration of NO in the aorta compared with the control lot. This decrease in NO concentration suggests that ADR causes endothelial dysfunction, which alters the ability of relaxing the endothelium by reducing the release of NO [29]. The ETDE administered to rats made hypertensive, has significantly reduced the concentration of NO in the aorta of rats made hypertensive and was normalized to the normotensive rats. Our result shows that ETDE have a protective role against endothelial dysfunction induced by ADR.

5. CONCLUSION

Our study showed that ETDE reduced DPPH, inhibits strongly the lipid peroxidation and reduces significantly the oxidative stress induced in hypertensive rats. Our results suggest that ETDE has antioxidant properties that could justify its use in the treatment of diseases caused by oxidative stress including high blood pressure.

ETHICAL APPROVAL

The experimental procedures and protocols used in this study were approved by the Ethical Committee of Health Sciences, University Felix Houphouët-Boigny. These guidelines were in accordance with the European Council Legislation 87/607/EEC for the protection of experimental animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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