

Evaluation of Antioxidant Activities of *Erythrococca anomala* Aqueous and Hydroethanolic Extracts from Leaves

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Abstract: *Erythrococca anomala* is an annual medicinal plant of the family Euphorbiaceae, widely used in traditional medicine in Côte d'Ivoire and sub-Saharan Africa as an anti-inflammatory, laxative and purgative. In the present work, the aim was to quantify the total phenols and flavonoids of the aqueous and hydroethanolic extracts of the leaves by the spectrophotometric method and to evaluate the antioxidant activity in vitro and in vivo of these extracts at the concentration of 200 mg / kg by the DPPH, ABTS⁺, FRAP and TBARS tests. Quantitative estimation of flavonoids and total phenols showed that the extract is rich in these compounds as the hydroethanolic extract. The aqueous extract (phenols, 85 ± 1.44 mg EAG / g and flavonoids, 28.33 ± 0.33 mg EQ / g) and the hydroethanolic extract (phenols, 69.17 ± 0.33 mg EAG / g and flavonoids, 23.23 ± 0.83 mg EQ / g) Evaluation of the antioxidant power by the free radical scavenging methods DPPH, FRAP iron reduction, ABTS⁺ and TBARS duction revealed that the aqueous extract had a higher antioxidant activity than that of the hydroethanolic extract, and neighboring Of that of quercetin and ascorbic acid.

Keywords: *Erythrococca anomala*, Euphorbiaceae, FRAP, TBARS, ABTS⁺, DPPH.

I. Introduction

The use of synthetic antioxidant molecules is currently being called into question due to the potential toxicological risks. Currently, new plant sources of natural antioxidants are being sought [1,2] because of their safety. Indeed, polyphenols are widespread natural compounds in plants that are increasingly important thanks to their beneficial health effects [3]. The role of natural antioxidants attracting more and more interest in the prevention and treatment of cancer, inflammatory and cardiovascular diseases [4]. They are also used as additives in the pharmaceutical and cosmetic industries [1]. Scientific methods have been developed for the extraction, identification and quantification of these compounds from medicinal plants [5, 6, 7].

Erythrococca anomala is a medicinal plant of the family Euphorbiaceae, widely used in traditional medicine in Cote d'Ivoire and sub-Saharan Africa. Phytochemical studies have revealed the presence of phenols, flavonoids, alkaloids, sterols and saponosides in leaves [8]. These secondary metabolites attract attention to play an essential role in the treatment of diseases [9]. Similarly, studies have shown that the leaves have anti-inflammatory activity [10].

In Côte d'Ivoire, the macerates of the leaves of this plant make it possible to fight against meningitis and malaria, while in Cameroon, decoctions and macerated leaves allow to treat dental pain; But used as laxatives and purgatives, make it possible to expel the worms; in Nigeria, bark is used against arthritis and rheumatism [11].

However, there is no information on the content of polyphenolic compounds and the antioxidant activity of leaves of *Erythrococca anomala*. This study aims to quantify the polyphenolic compounds by the spectrophotometric method, to evaluate the antioxidant activity in vitro and in vivo by the method of the trapping of the free radical DPPH, reduction of the cation ABTS⁺ the tests of FRAP and TBARS of the aqueous and hydroethanolic extracts of leaves of *Erythrococca anomala*.

II. Materials and Methods

2.1. Plant material

Erythrococca anomala plant was collected in January 2015 from Yakasse-me area in the Department of Adzopé (Côte d'Ivoire). The plant was identified and authenticated by the Laboratory of Botanic, University Felix Houphouet Boigny. The authentically identified plant material (leaves) was washed and dried for 3-4 days in laboratory at room temperature. It was powdered and subjected to different extraction procedures.

2.2. Animal material

Wistar albinos rats weighing 190-200 g of each sex kept for three weeks at the laboratory animal home of UFR Pharmaceutical and Biologic Sciences, University of Felix Houphouët Boigny, Côte d'Ivoire were used. The animals were maintained under standard housing conditions : temperature ($27^{\circ}\pm 1^{\circ}\text{C}$), humidity (55-60%), light/dark cycle (12:12 h) and had free access to standard rodent pellet diet and water ad libitum.

2. Methods

2.1 Preparation of extracts

The aqueous extract is prepared from 100 grams of *Erythrococca anomala* leaf powder in one liter of boiling distilled water for fifteen minutes.

The solution thus obtained is filtered on hydrophilic cotton and then under vacuum with ordinary filter paper. The collected filtrate was placed in an oven at 40°C . A dark brown dry powder was obtained for the total crude aqueous extract of *Erythrococca anomala*.

The Guédé-Guina method [12] was used to obtain the 70% *Erythrococca anomala* hydroethanolic extract. A 70% hydroethanolic solution (ETOH / H₂O, 70: 30) was used for the preparation of The hydroethanolic extract of *Erythrococca anomala* in a vial. One liter of the hydroethanolic solution and 100 g of *Erythrococca anomala* powder were used for this purpose. The resulting mixture was homogenized with a magnetic stirrer for 24 hours. The homogenate is filtered on hydrophilic cotton then under vacuum. The collected filtrate was concentrated in a rotary evaporator and then placed in an oven at 40°C . for complete drying. The 70% hydroethanolic extract of *Erythrococca anomala* appears in the form of a dark green paste after drying.

2.2. Quantification of polyphenolic compounds

2.2.1. Spectrophotometric assay of phenols

Wood's method [13] was used for the determination of total phenols in aqueous and hydro-ethanolic extracts of leaves of *Erythrococca anomala*. A volume of 2.5 mL of diluted Folin-Ciocalteu reagent (1/10) was added to 30 μL of extract. The mixture was held for 2 minutes in the dark at room temperature, the 2 mL of calcium carbonate solution (75 g L^{-1}) was added.

The mixture was then placed for 15 minutes in a water bath at 50°C . and then cooled rapidly. The absorbance was measured at 760 nm, with distilled water as white. A calibration line was made with gallic acid at different concentrations. The analyzes were carried out in triplicate and the concentration of phenols was expressed in grams per liter of gallic acid equivalent extract (g.L^{-1} , Eq AG).

2.2.2. Spectrophotometric determination of flavonoids

The Marinova method [14] was used for the determination of total flavonoids in aqueous and hydroethanolic extracts of leaves of *Erythrococca anomala*. Into a 25 mL flask, 0.75 mL of 5% (w / v) sodium nitrite (NaNO₂) was added to 2.5 mL of extract. The mixture was supplemented with 0.75 mL of 10% (w / v) aluminum chloride (AlCl₃) and then incubated for 6 minutes in the dark. After incubation, 5 mL of sodium hydroxide (1N NaOH) was added and the volume was then made up to 25 mL. The mixture was stirred vigorously before being assayed to the UV-visible spectrophotometer. The reading was made at 510 nm. The trials were carried out in triplicate. The flavonoid content was expressed in grams per liter of quercetin equivalent extract.

2.3. Evaluation of antioxidant activity

2.3.1. Investigation of antioxidant activity *in vitro*

2.3.1.1. Preparation of ABTS

This method is based on the capacity of the compounds present in the aqueous and hydroethanolic extracts of the leaves of *Erythrococca anomala* to reduce the cation radical ABTS + (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid). The test was performed according to the method described by Choong [15]. Radical-cation ABTS⁺ was produced by reaction of 8 mM ABTS (87.7 mg in 20 mL of distilled water) and 3 mM potassium persulfate (0.0162 g in 20 mL distilled water) in a 1: 1 (v / v) ratio.

The mixture was then incubated in the dark at room temperature for 12-16 hours. This ABTS + solution was diluted with methanol so as to obtain a solution whose absorbance was 0.7 ± 0.02 at 734 nm. Thus, a test sample of 3.9 mL of this diluted ABTS⁺ solution was added to 100 μL of the compound to be tested.

After stirring, the mixture was incubated for 6 minutes in the dark ($T = 30 \pm 2^{\circ}\text{C}$). The residual absorbance of the ABTS + radical was then measured at 734 nm on the UV-visible spectrophotometer and should be between 20% and 80% of the absorbance of the blank. The trials were carried out in triplicate and the results were expressed in μmol Trolox equivalent per liter of extract ($\mu\text{mol TE / L}$).

A calibration line was made with the following Trolox concentrations : 0.375 μM ; 0.5 μM ; 0.625 μM ; 1 μM ; 1.125 μM , 1.375 μM and 1.5 μM and the inhibition rate (% I) of ABTS⁺ was expressed as follows:

$$\% I = [(A_0 - \text{Abs extract}) / A_0] \times 100$$

A₀ = diluted ABTS absorbance.

Abstrand = absorbance ABTS diluted + sample

This line allowed to express the antioxidant activity of the various extracts as follows: Concentration or Reductive Capacity of the cation ABTS (μM eq Trolox) = (% I x fd) / (4.99 x 10)

2.3.1.2. Study of the antiradical activity by the trapping of the DPPH radical

The antiradical activity of extracts of leaves of *Erythrococca anomala* was measured by the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) test according to the Parejo method [16] with some modifications. A range of concentrations (0-200 μg / mL) of aqueous and hydroethanolic extracts of leaves of *Erythrococca anomala* or Quercetin (reference antioxidant) is prepared in ethanol / water (70/30) (v / v) .

A volume of 100 μL of this solution is mixed with 3.9 mL of DPPH (70 μM) prepared in methanol. After homogenization, the mixture is incubated at room temperature (25 ° C.) to Shelter from light. After 15 minutes of incubation, the absorbance is read at 517 nm against a "white" which contains only methanol.

The percentage inhibition of the DPPH radical is calculated according to the following equation :

$$\text{Inhibition of DPPH (\%)} = (A_0 - A_{\text{extract}}) / A_0 \times 100.$$

A₀ = absorbance (control)

A extract = absorbance (extract)

IC₅₀, which is the concentration of leaf extract or Quercetin responsible for 50% inhibition of DPPH radicals, is determined by projection from 50% on the graph representing the percentage inhibition of DPPH as a function of the concentrations Extracts and Quercetin. (% DPPH inhibition = f (extract concentrations).

2.3.2. Study of antioxidant activity *in vivo*

2.3.2.1. Induction of oxidative stress by carrageenan

Oxidative stress was produced by injection of carrageenan at the left hind paw of rats. Wistar rats were fasted for 16 het were weighed before being used for the experiment. They were subdivided into 5 groups of 6 rats with an average weight of 130 g. Group I received saline (control), group II received vitamin C (reference molecule), groups III, IV and V respectively received aqueous and hydroethanolic extracts from the leaves of *Erythrococca anomala* (200 mg / Kg body weight) and NaCl + carrageenan. All the animals received 1 h after treatment 0.2 ml of 1% carrageenan suspension in the left hind foot subplant area. The blood of the rats is taken 5 hours later in heparinized tubes. This blood is then centrifuged and the serum is used for the study of antioxidant activity.

2.3.2.2. Determination of TBARS

The TARSB (Thiobarbituric Acid Reactive Substances) assay in aqueous and hydroethanolic extracts of the leaves of *Erythrococca anomala* was carried out according to the Satho method [17]. To prepare the serum proteins, 2.5 mL TCA 20% (m / V) was added to 0.5 mL of rats serum, which was centrifuged at 1500 g for 10 min. Next, 2.5 mL of sulfuric acid and 2 MI TBA (0.2%) were added to the sediment. This mixture reaction was then shaken and then shaken and then incubated for 30 minutes in a boiling bath. After addition of 4 mL of n-butanol, the solution was centrifuged, cooled and the absorbance of the supernatant read at 352 nm. The calibration curve was obtained using different concentrations of 1, 3,3 tetramethoxypropane (0.0, 1.875, 2.380, 4.760, 9.530, 19.050, 38.110) as the standard for determining the concentration of TBA-MDA adducts in the sample.

2.3.2.3. Determination of total antioxidant power : the FRAP test

The determination of the total antioxidant power of the aqueous and hydroethanolic extracts of leaves of *Erythrococca anomala* present in the serum according to the method of **Benzie** and **Strain [18]** is based on the measurement of the ability to reduce the Fe³⁺ ion to Fe²⁺ ion by the FRAP test (FerricReducing Ability of Plasma). The FRAP test measures the variation in absorbance at 593 nm of a blue complex (FeII-tripyridyltriazine (TPTZ) by the action of electron-donating antioxidants. The FRAP reagent is composed of 300 mmol.L-1 of Acetate buffer, pH = 3.6, 10mmol.L-1 TPTZ in 40 mmol · L⁻¹ HCl and 20 mmol · L⁻¹ FeCl₃, 6 H₂O in the ratio of 10: 1: 1.

Thus, 10 μL of serum was added to 300 μL of FRAP reagent (freshly prepared and preheated at 37 ° C.) in a test tube. After 10 min incubation at 37 ° C, the absorbance of the blue complex is read against a blank (300 μL of FRAP reagent + 10 μL of distilled water) at 593 nm. The Fe²⁺ standard solutions in the concentration range from 100 to 1000 mmol.L⁻¹ were prepared from ferrous sulfate (FeSO₄, 7H₂O) in distilled water. The data were expressed in mmol of ferric ions reduced to the ferrous form per liter (FRAP value).

2.4 Statistical Analysis

The values expressed as mean \pm SEM from 6 animals. The results were subjected to statistical analysis by using one way ANOVA followed by Dunnett's test to significant, p values less than 0.05 were considered significant.

III. Results

3.1.1. Quantification of antioxidant compounds (phenols and flavonoids) *in vitro* of aqueous and hydroethanolic extracts of leaves of *Erythrococca anomala*

The amounts of total polyphenol in the aqueous and hydroethanolic extracts of *Erythrococca anomala* leaves are shown in **Table 1**. The aqueous extract had more phenols (85 ± 1.44 mg EAG / g) and flavonoids (28.33 ± 0.33 mg EQ / g) than the hydroethanolic extract (69.17 ± 0.83 mg EAG / g) of phenols and flavonoids ($23.23 \pm 0.83 \pm 0.33$ mg EQ / g). The polyphenolic compounds were present both in the aqueous extract and in the hydroethanolic extract.

Table 1 : Total polyphenol and total flavonoid content of the aqueous and hydroethanolic extracts of the leaves of *Erythrococca anomala*.

Extracts	Content phenols gallic acid (mg EAG / g)	Flavonoid Content Quercetin (mg EQ / g)
Aqueous extracts	85 \pm 1.44	28.33 \pm 0.33
Hydroethanol extract	69.17 \pm 0.83	23.23 \pm 0.83

Values represent the average \pm SD, (n = 3) ; (EAG) Gallic acid equivalent; (EQ) Equivalent quercetin.

3.1.2. Reductive capacity of the ABTS⁺ ion of aqueous and hydroethanolic extracts of the leaves of *Erythrococca anomala*.

The antioxidant activities of the extracts are given in **Table 2**. The extract has an activity equal to 34.45 ± 3.13 μ mol Trolox equivalent / L whereas that of the hydroethanolic extract of 26.82 ± 1.73 μ mol Equivalent Trolox / L. The reducing capacity of the ABTS⁺ ion of the aqueous extract is greater than that of the hydroethanolic extract.

Table 2 : Reductive capacity of the ABTS ion of the aqueous and hydroethanolic extracts of the leaves of *Erythrococca anomala*.

Extracts	Antioxidant activity expressed in μ mol Trolox equivalent / L
Aqueous extracts	34.45 \pm 3.13
Hydroethanolic extract	26.82 \pm 1.73

Values represent the average \pm SD, (n = 3).

3.1.3. Anti-freeze activity by the DPPH test

The IC₅₀ values of the extracts determined graphically by the linear regressions of the graphs lined against that of quercetin (5.75μ g / mL) are respectively 10.5μ g / mL and 21.5μ g / mL for aqueous and hydroethanolic extract **Fig 1**.

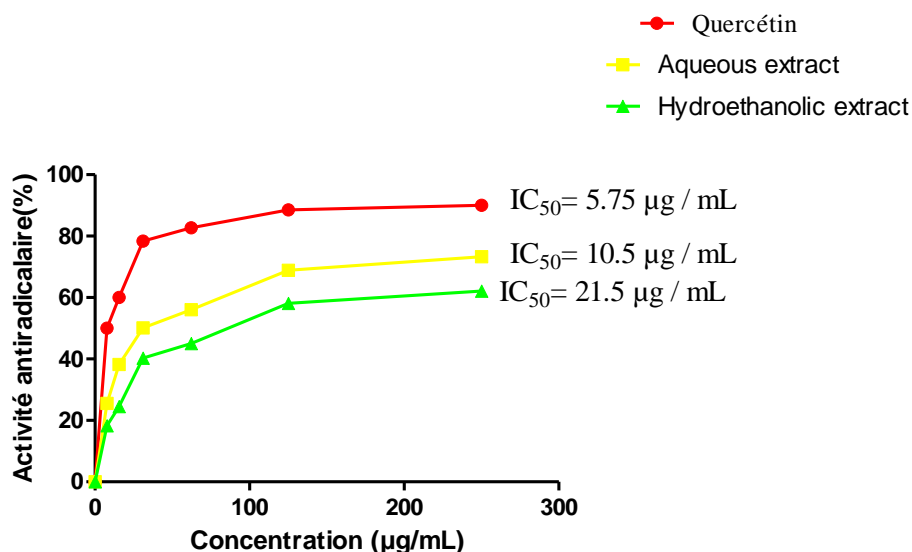


Fig 1 : Anti-radical activity of aqueous and hydroethanolic extracts of leaves of *Erythrococca anomala*

3.2. Evaluation of the in vivo antioxidant activity of aqueous and hydroethanolic extracts of leaves of *Erythrococca anomala* : Total antioxidant power by TBARS and FRAP tests

The values obtained with the TBARS and FRAP test are shown in **Table 3**. The values of the aqueous extract and the vitamin C are very high compared to the NaCl control with the TBARS test with $p < 0.001$. More over with the test of FRAP and TBARS the antioxidant power of the vitamin C and the aqueous extract is much higher compared to the control. However vitamin C recorded the greatest value with both methods.

Table 3 : *In vivo* antioxidant activity of leaves of *Erythrococca anomala* by TBARS and FRAP tests

Extracts	TBARS (%)	FRAP ($\mu\text{mol de fer II/L}$)
NaCl + carrageenan	25.83 \pm 0.98	3.8 \pm 0.75
Hydroethanolic extract	22.67 \pm 0.81	7.2 \pm 0.75
Aqueous extracts	9.16 \pm 1.16 ***	10 \pm 1.00***
Vitamine C	7 \pm 1.03 ***	12 \pm 0.75***
NaCl	8.5 \pm 0.83	6.3 \pm 0.52

Each value is mean \pm SEM, N= 6 rats, the data was analyzed by using One Way ANOVA followed by Dunnett's test ** $p < 0.001$, where Extracts and control were compared with NaCl.

IV. Discussion

Among the recognized biological potentialities of plants, antioxidant activity is of growing interest because of the important roles played by antioxidant compounds found in plants, in the treatment and prevention of oxidative dysfunction diseases [19, 20,21].

The spectrophotometric method was used in this study to quantify the polyphenols. The antioxidant activity was evaluated by trapping of free radicals such as the method of FRAP, TBARS, and the method using the DPPH radical and the cationic radical ABTS⁺.

Aqueous and hydroethanolic extracts (200 mg / kg bw) showed significant antioxidant activities in vitro by inhibition of the DPPH radical and reduction of ABTS⁺ cation in comparaisn with vitamin C and quercetin.

In addition, tests of FRAP and TBARS *in vivo* revealed the reductive and inhibitory activities of the two extracts in comparison with that of vitamin C. The antioxidant capacity of plant extracts is largely dependent on the composition of these extracts. The reducing power of leaves of *Erythrococca anomala* is probably due to the presence of hydroxyl groups in the polyphenolic compounds (phenols and flavonoids) which can serve as electron donors, therefore antioxidants are considered oxidant reducing agents and inhibitors

[22]. A few previous studies have also shown that the reducing power of a compound can serve as a significant indicator of its potential antioxidant activity [23,24] The agreement between the DPPH, ABTS⁺, FRAP and TBARS radical tests is that the four tests retain the aqueous extract as a powerful antioxidant.

Water is a solvent which can extract the majority of chemical constituents such as water-soluble and water-soluble polyphenolic compounds of anti-free radical properties.

V. Conclusion

The evaluation of the antioxidant activity of the aqueous and hydroethanolic extracts of the leaves of *Erythrococca anomala* par the in vitro tests of the DPPH radical, ABTS⁺ cation and *in vivo* the tests of FRAP, TBARS demonstrated the reducing power and inhibitors of these extracts . In this study, aqueous and hydroethanolic extracts appear to be of real and potential interest by their antioxidant activities that have been established both in vitro and *in vivo*. By these tests, the aqueous excipient revealed the greatest antioxidant power close to those of quercetin and ascorbic acid.

The study of the polyphenolic composition of the various extracts elucidates the cause of the great antioxidant and antiradical activity of the aqueous extract relative to the hydroethanolic extract.

Ethical approval

The experimental procedures and protocols used in this study were approved by the Ethical Committee of Health Sciences, University Félix Houphouet-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.

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