

Evaluation of Some Secondary Metabolites and Determination of the Antioxidant Potential of Different Extracts from the Plant of *Pteridium aquilinum*

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Abstract

The present study aims to make an evaluation of some secondary metabolites and determination of the antioxidant potential of *P. aquilinum* plant extracts obtained by means of a simple and rapid TLC method. The latter revealed the presence of terpenes, sterols, steroids, flavonoids, polyphenols, saponins, sugars and amino acids. The evaluation of the potential antioxidant was assessed on phenolic and flavonoid compounds. These compounds' dosages revealed different levels, but the highest antioxidant activity was found in the hydro-ethanol extract followed by the aqueous extract. Among the two families of evaluated antioxidants, phenolic compounds were found to be higher in the hydro-ethanolic extract (75.18 mgEAG/gM_s), followed by the aqueous extract (66.78 mgEAG/gM_s) and lower in the ethanolic extract (12.39 mgEAG/gM_s). Whereas flavonoids, less significantly elevated, showed values of 2.58 mgECa/gM_s for the hydro-ethanolic extract, 2.24 mgECa/gM_s for the aqueous extract and 1.58 mgECa/gM_s for the ethanolic extract. However, the antiradical activity was also evaluated. Contrary to the antioxidant activity, the most important antiradical activity was observed on the hydroethanolic extract with a rate of 3.61 mg/mL, then a weak activity on the aqueous and ethanolic extracts respectively 6.18 mg/mL and 15.81 mg/mL, then less important on the aqueous

and hydro-ethanolic extracts respectively at levels of 6.18 mg/mL and 3.61 mg/mL.

Keywords

Evaluation, Extracts, Secondary Metabolites, Potential Antioxidant, *Pteridium aquilinum*

1. Introduction

Higher plants have the ability to synthesize, through complex metabolic pathways, also called secondary metabolites. These compounds are used by plants for various functions especially in response to biotic and abiotic stresses they may undergo [1]. These metabolites are listed in one of three classes: polyphenols, alkaloids and terpenoids. Many studies have revealed the bioactivity of these molecules in antitumor, antiviral, antimicrobial, antioxidant, anti-inflammatory activities, etc. Plants containing these metabolites can be used in therapeutic, pharmaceutical, cosmetological and food fields [1] [2].

Antioxidants, which are secondary metabolites, appear to be nowadays the keys to longevity and our allies in the fight against modern diseases. They are protective elements that have an extreme variety of structures and biological activities and act as free radical scavengers. Therefore, an antioxidant is defined as a substance capable of preventing or slowing down the oxidation of other molecules [3].

Free radicals are produced daily in large quantities by the organism via oxygen, which is essential to our life, and they are very reactive compounds containing a single electron and are necessary for vital mechanisms [4]. But they become harmful when they are in excess and induce some damage to the structure of proteins, lipids [5], nucleic acids [6] by causing oxidative stress that contributes to the process of accelerated cellular aging and the development of many human diseases such as cardiovascular disease, cancer, arteriosclerosis [6], diabetes, Alzheimer's disease, rheumatism [7] [8].

According to [9], in order to avoid the serious consequences of oxidative stress, it is necessary to maintain the balance between oxidants and antioxidants, in order to preserve the optimal physiological performance of the organism. The adequate consumption of antioxidants is essential. Considered as defense systems, they can be endogenous or exogenous, of nutritional origin.

Endogenous antioxidants are produced by our body. These include enzymatic systems such as superoxide dismutases, catalases, glutathione peroxidases and thioredoxin systems, which are known to be very effective in detoxifying ROS (reactive oxygen species). The main non-enzymatic antioxidants present in the human body are glutathione, bilirubin, sex hormones, uric acid, coenzyme Q, melanin, α -tocopherol and lipoic acid [10]. They are permanently present in our body but their quantity decreases with age [11].

In nature, and particularly in the plant world, plants contain many bioactive substances that have antioxidant properties. For this reason, it is very interesting to search and identify natural antioxidants from plants [12] [13]. Moreover, according to [9], numerous epidemiological and clinical studies confirm the undeniable role of regular consumption of fruits and vegetables in reducing the risk of cancers and chronic diseases, especially cardiovascular diseases.

For a very long time, medicinal plants were the main recourse for human beings for the manufacture of pharmaceutical remedies [14]. An attitude that may also be related to the ancestral culture and civilization, which is based entirely or partially on herbal medicine because of the effectiveness, accessibility and availability of medicinal plants [15].

The aim of the present study is to evaluate the antioxidant activity of the species *Pteridium aquilinum* L. Kuhn. Thus, this plant's analysis was based on the partial scanning of secondary metabolites followed by the determination of total polyphenols and flavonoids of three different extracts (aqueous, hydro-ethanolic and ethanolic) *in vitro* using different methods.

2. Materials and Methods

2.1. Plant Material

The fern plant was purchased in the local market (the tsieme) in one of the north-western districts of Brazzaville. The part used in this study is made up of fronds (Figure 1) maintained in the shelter of the light and at room temperature (25°C) during approximately five (05) days. The dry matter was then crushed with an apparatus of type IKA-WERKE GmbH-CO-KG, D-79219 Staufen, equipped with a sieve of granulometry 0.25 mm.

2.2. Methods

2.2.1. Preparation of Extracts

The chemical screening of *P. aquilinum* on thin layer was performed on the ethanolic extract.

Concerning the determination of total polyphenols and flavonoids, the different extracts were obtained by mixing 40 g of the plant material in 2×200 mL of different organic solvents for the preparation of aqueous, hydro-ethanolic in



Figure 1. (a) Rhizomes of fern (*P. aquilinum*); (b) Fronds of *P. aquilinum*.

the proportions 50% (v/v) and ethanolic in the proportions 50% (v/v) respectively. The mixture was macerated under stirring for 48 h and then filtered through a Wattman filter paper. The filtrate obtained was concentrated to dryness at 50°C under reduced pressure using a rotary evaporator model N-1 (Eyela, Tokyo Rikakikal, Co., Ltd., Japan). This filtrate was placed in an oven at 25°C for 24 h and then stored in a cool place (+4°C) waiting to be analyzed. The alcohol used is Cooper brand preparatory at 90% vol.

2.2.2. Preparation of Assay Solutions at Different Dilutions

A series of glass tubes were used to prepare the solutions of different extracts with the corresponding solvents. 40 mg of ethanolic extract, 80 mg of hydro-ethanolic extract and 160 mg of aqueous extract respectively were measured to prepare the assay solutions by adding 2 mL of solvent (Ethanol, Water-Ethanol and Water) in each of the tubes. Everything was mixed under magnetic stirring for a few moments and the stock solutions for each extract were obtained.

From the stock solutions, we also prepared the subsequent solutions for each extract by 1/10 dilution. We prepared a total of 4 (S1 to S4) of each extract from which we obtained 12 following solutions.

3. Thin Layer Chromatography Method: TLC

3.1. Chemical Screening by TLC

The different extracts were subjected to analysis by Thin Layer Chromatography (TLC), performed on silica 60 F254 plates (Merck). Different elution systems were used according to the types of families sought. After development, the plates were examined under the UV lamp at 254 nm and 365 nm. The spots were then revealed by conventional detection reagents prepared according to standard methods [16].

3.2. Thin Layer Chromatography of Antioxidant Activity

Quantitative identification of substances with antioxidant activity was performed according to the “bioautography” method [17] by thin layer chromatography where antioxidant activity was revealed with DPPH, according to [18]. TLC was performed on silica gel chromatographic plate 60F254 on 20 cm × 20 cm aluminum foil support of Merck brand.

TLC was performed in normal phase on aluminum plates with the solution of ethyl acetate/formic acid/water in proportions 9/0.5/0.5. The chromatogram obtained was revealed by spraying with the Node solution (0.5 g of 2 amino diphenyl borinate + 0.5 g of PEG400 + 100 mL of ethanol).

Observation of the plates was performed in UV-visible and UV at 366 nm, before and, in some cases, after revelation with the appropriate reagents. The UV lamp used is a CAMAG lamp with a wavelength ranging from 254 to 366 nm.

3.3. Polyphenol Analysis

The total polyphenol content of the different extracts of the *P. aquilinum* plant

was determined according to the Folin-Ciocalteu method. For this purpose, to each 0.1 mL of each extract (aqueous, hydro-ethanolic and alcoholic) is added, 0.9 mL of distilled water, followed by the addition of 0.9 mL of (1N) Folin-Ciocalteu reagent. Immediately thereafter, 0.2 mL of Na₂CO₃ (20%) was added. The resulting mixture was incubated at room temperature of 25 °C for about 40 minutes in the dark.

The absorbance was measured with a spectrophotometer UV-Visible Spectrophotometer, model Gd-752n at 725 nm against a methanol solution used as blank. The results obtained were expressed as mg gallic acid equivalent per gram of dry matter (EGA/g Ms).

3.4. Total Flavonoids Analysis

The total flavonoid content of the different extracts of *P. aquilinum* was obtained using Aluminium Trichloride (AlCl₃) [19]. In a 100 mL flask was successively introduced 250 µL of each extract (aqueous, hydroethanolic and alcoholic) 1 mL of distilled water, 7.5 µL of NaNO₂ (5%). The mixture was allowed to stand for 5 minutes. Then 75 µL of AlCl₃ (10%), was added before letting it stand again for 6 min. Then 500 µL of (1N) NaOH and 2.5 mL of distilled water were added successively to the mixture. The analyses were performed at 25 °C.

The following reagents: NaOH, NaNO₂, Na₂CO₃, ethyl acetate and formic acid are all from Merck.

The absorbance was measured by UV-visible spectrophotometer UV-Visible Spectrophotometer, model Gd-752n at 510 nm and the results were expressed as mg catechin equivalent per gram of dry matter (ECa/g Ms).

3.5. Evaluation of the Anti-Free Radical Activity of Different Extracts

3.5.1. Method Using DPPH

The antioxidant activity of each extract was measured using DPPH radical.

The evaluation of the anti-free radical activity was performed using 5 mL of the solution of 1.1-diphenyl-2-picrylhydrazyl (DPPH at 10 mg in 250 mL of ethanol) and 100 µL of each extract diluted to concentrations ranging from 1.25 to 20 or even 40 mg/mL, all mixed in EDTA glass tubes. After 30 minutes of incubation in the dark, the free radical scavenging activity was measured by spectrophotometer at 517 nm in the dark [20]. The percentage of inhibition was calculated by the following relation:

With D.O_{extracted}: 0.727, with D.O_{blank}: 0.788:

$$I\% = \frac{D.O_{\text{blank}} - D.O_{\text{extract}}}{D.O_{\text{blank}}} \times 100$$

The IC₅₀ parameter (50% inhibitory concentration) is defined as the concentration of the substrate that causes the loss of 50% of DPPH activity. The antioxidant power is determined in such a way that an amount of the extract of a certain concentration neutralizes 50% of the DPPH radical. In order to compare

the extracts between them, this index is obtained either by deduction from the curves of the variation of the percentage of inhibition I% or calculated graphically by the formula of the regression of the percentages of inhibition according to different concentrations of the extracts tested with the help of the XL Stat Pro 7.5. The value of the antiradical activity, such as $y = 50\%$, corresponds to the inhibitory concentration IC₅₀ of the studied extract [20] [21] [22].

The results expressed as IC₅₀ were deduced from the data presented from the variation of the percentage of inhibition I% as a function of the concentration of each extract. It should be remembered that the smaller the IC₅₀ value, the greater the antioxidant activity of the extracts [23] [24].

The following reagents: DPPH, Folin-Colciateu, Gallic acid, Aluminum trichloride, Quercetin, Rutin, Caffeic acid and Chlorogenic acid are all from Sigma

3.5.2. Statistical Studies

The experiments were done in triplicate, the results were presented by the mean with its standard deviation. The analyses of variance were performed by the statistical software XL Stat Pro 7.5. The determination of significance levels was performed by the Anova test. Differences were considered significant at $P < 0.05$.

4. Results and Discussion

4.1. Chemical Screening by TLC

Chemical screening of *P. aquilinum* frond extracts revealed the presence of large families of chemical compounds (Figure 2). Each of the families appear as spots of different color.

The green, yellow, orange, blue, pink and purple spots observed on the chromatographic plates and in UV/visible spectroscopy, correspond to different secondary metabolics such as terpenes, sterols, steroids, flavonoids, polyphenols, saponins, sugars and amino acids.

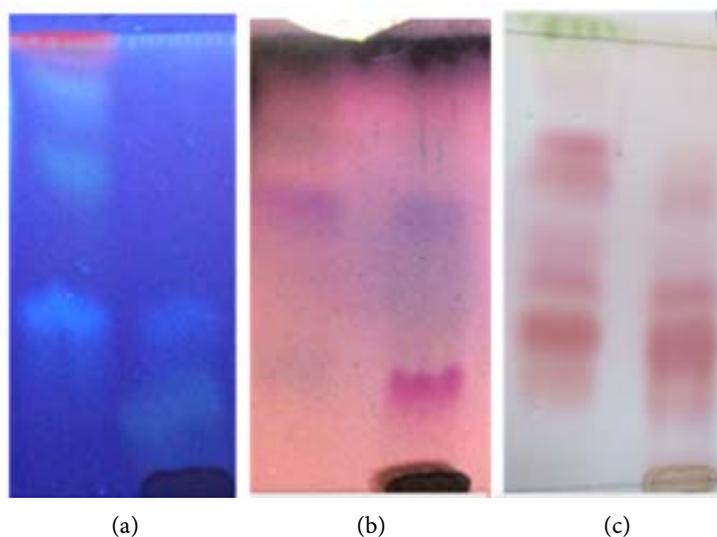


Figure 2. Research of some secondary metabolites by TLC.

The blue, bluish-white spots observed on the chromatograms under UV/365 nm (**Figure 2(a)** and **Figure 2(b)**) revealed by the node reagent or aluminum chloride, characterizes the presence of polyphenols. Flavonoids are also revealed under UV/366 nm under various fluorescent white, pink, orange and green colors (**Figures 2(a)-(c)**).

Iron trichloride is the appropriate developer for tannins, the latter appear on the plate after FeCl_3 development as a black or blue spot. Thymol and sulfuric vanillin reveal all glycosylated compounds [25] [26].

Some previous works have highlighted the presence of alkaloids, flavonoids, polyphenols and tannins in the fronds of *P. aquilinum* L. Kunh [27].

For this study, the focus was on the antioxidant properties of *P. aquilinum* extracts, especially the two families (polyphenols and flavonoids).

4.2. Thin Layer Chromatography of Antioxidant Activity

Figure 3 shows the presence of yellow-pale spots on a purple and blue background of the hydro-ethanol extract of *P. aquilinum*. This indicates that this extract, compared to the other two, contains phytochemicals likely to trap free radicals. Indeed, the chromatographic profile on TLC plate revealed at the node and observed at UV (365 nm) revealed on the one hand the blue, bluish white spots which remind the presence of polyphenols, and on the other hand, the white, pink, orange and green spots are characteristic of flavonoids.

By contrasting the chromatographic profiles of the phytochemical screening and that of the antioxidant activity assay (**Figure 3**), the correspondence between the active zones and the phytochemicals responsible for this activity was

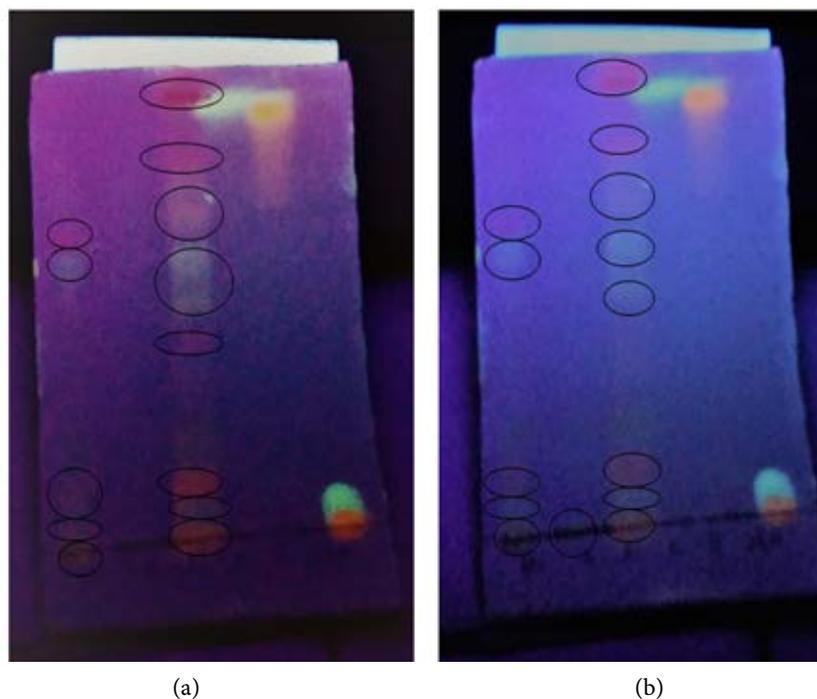


Figure 3. Chromatographic profiles of extracts and some reference compounds.

established. In the chromatogram of the hydro-ethanolic extracts (**Figure 3**), the yellow spots would correspond to the antioxidant activity of flavonoids and polyphenols.

The observation of the TLC plates shows yellow, yellow-orange fluorescences corresponding to the reference compounds Quercetin (Qr) and Caffeic Acid (Ac-Caf). Yellow and green fluorescence which can correspond to the reference compound Chlorogenic acid (Ac.Chl). Orange fluorescence that may correspond to the reference compound Rutin (Rut) is observed. According to [28] [29], the colored fluorescences observed for the different reference compounds (Qr, Rut, Ac.Caf, Ac.Chl) are characteristic of flavonoids and polyphenols.

4.3. Phenols and Flavonoids Content

The determination of total phenols and flavonoids (**Figure 4**) in the different extracts of *P. aquilinum* was done using separately the colorimetric methods (Folin-Ciocalteaux and Aluminium Trichloride).

The quantitative analysis of total polyphenols and flavonoids shows that the hydro-ethanolic and aqueous extracts are quantitatively richer than the ethanolic extract. The contents of polyphenols in the extracts are respectively 75.18 mgEAG/gM_s for the hydro-ethanolic extract, 66.78 mgEAG/gM_s for the aqueous extract and 12.39 mgEAG/gM_s for the ethanolic extract against 2.58 mgECa/gM_s for the flavonoids on the hydro-ethanolic extract, 2.24 mgECa/gM_s for the aqueous extract and 1.58, mgECa/gM_s for the ethanolic extract. It can be seen that all the extracts of *P. aquilinum* are rich in polyphenols and present low levels of flavonoids.

These differences in content between the different compounds can be explained by the fact that the total polyphenols include flavonoids and other compounds.

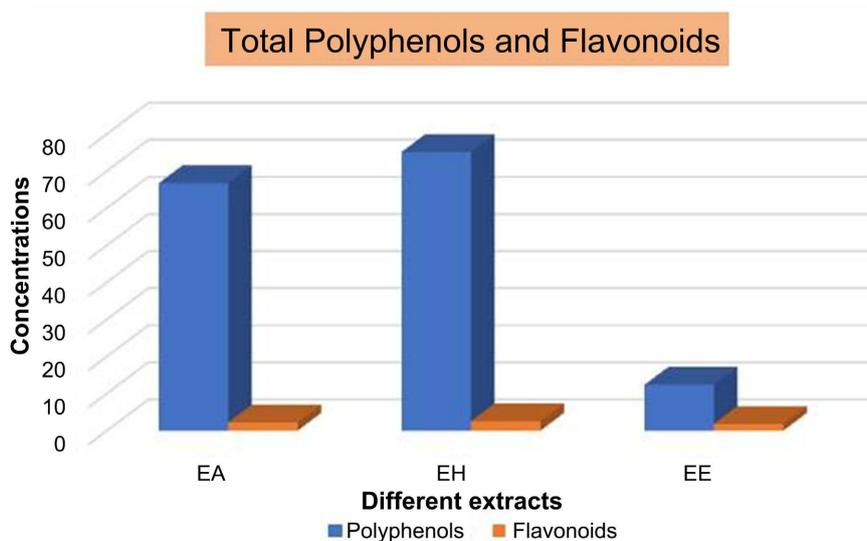


Figure 4. Dosage of total polyphenols and flavonoids in aqueous, hydro-ethanolic and ethanolic extracts.

It is also noted that the hydroalcoholic and aqueous extracts are quantitatively richer in phenolic compounds that is, that solvents (water and water-ethanol mixture) extract better polyphenols compared to other mixtures [25] [26].

The literature reports that, it is in the alcoholic extracts that we find more phenolic compounds [30] [31] while for our case, it is in the hydroethanol and aqueous extracts that we found more phenolic compounds. The high level of these compounds in the hydroethanolic and aqueous extracts leads us to deduce that water and the water/plus alcohol mixture are solvents for better extraction of these compounds. This is due to the capacity of alcohol to inhibit the action of the polyphenol oxidase which causes the oxidation of polyphenols in plant tissues [32] for the case of solvent mixture.

It can also be noted that the stationary phase used (polyamide 6-Fluka) allowed to enrich these extracts in poly-phenolic compounds. The high contents of total polyphenols and flavonoids obtained in the present study, could be justified by the very clear evidences observed by thin layer chromatography (TLC) and the presence of these metabolites reported by several authors in the plant [33].

4.4. Anti-Radical Activity of the Different Extracts

4.4.1. Percentage of DPPH Radical Inhibition

The results of the anti-free radical activity of the different extracts on DPPH are presented in the series of **Tables 1-3**. **Tables 1-3** show that at a low concentration of 1.25 mg/ml, the hydro-ethanolic, aqueous and ethanolic extracts, present

Table 1. Anti-radical activity of the hydro-ethanolic extract of *P. aquilinum*.

Hydroethanolic Extract					
Concentration	20 mg/ml	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml
Optical Density (O.D.)	0.25	0.21	0.37	0.70	0.92
Percentage of Inhibition (%)	69.76	60.09	40.46	24.74	14.51

Table 2. Anti-radical activity of the aqueous extract of *P. aquilinum*.

Aqueous Extract					
Concentration	20 mg/ml	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml
Optical Density (O.D.)	0.33	0.43	0.64	0.81	0.92
Percentage of Inhibition (%)	76.74	80.84	65.21	34.60	20

Table 3. Anti-free radical activity on ethanolic extract of *P. aquilinum*.

Ethanolic Extract					
Concentration	20 mg/ml	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml
Optical Density (O.D.)	0.53	0.74	0.87	0.96	1.002
Percentage of Inhibition (%)	50.88	31.63	18.61	9.95	6.79

percentages of reduction of DPPH, respectively 14.51%, 20% and 6.79% but at high concentrations from 20 mg/ml. We note in the same order 69.76%, 76.74% and 50.88%. It can be seen that the values of the anti-free radical activity increase with the concentration in the extracts.

4.4.2. 50% Inhibitory Concentrations

The results of the anti-free radical activity were also expressed using the IC₅₀ parameter. The IC₅₀ is inversely related to the antioxidant capacity of a compound, so the lower the IC₅₀ value, the higher the antioxidant activity of a compound.

The IC₅₀ found with the ethanolic extract of *P. aquilinum* (15.81 mg/mL) is significantly higher ($P < 0.05$) than that found with the aqueous extract (6.18 mg/mL). On the other hand, the IC₅₀ value found with the hydroalcoholic extract (3.61 mg/mL) is significantly lower ($P < 0.05$). These results are in agreement with those found by [30], where they stated that both extracts had high inhibitory power on *P. aquilinum* plant.

These low values of the inhibitory concentration 50% (IC₅₀) of the hydro-ethanolic and aqueous extracts show that they are endowed with greater antioxidant power than that of the ethanolic extract and this explains why water and the alcohol/water mixture remains the best extraction solvent for this study.

This strong inhibition of free radicals by ethanolic and aqueous extracts (**Figure 5**) could be justified by their high concentrations of phenolic compounds which are known to be potent compounds with free radical reducing power [34] [21].

We can also note that the anti-radical activity is the opposite of the antioxidant activity. Indeed, the polyphenolic compounds are known as powerful compounds having a reducing power of the free radicals [21].

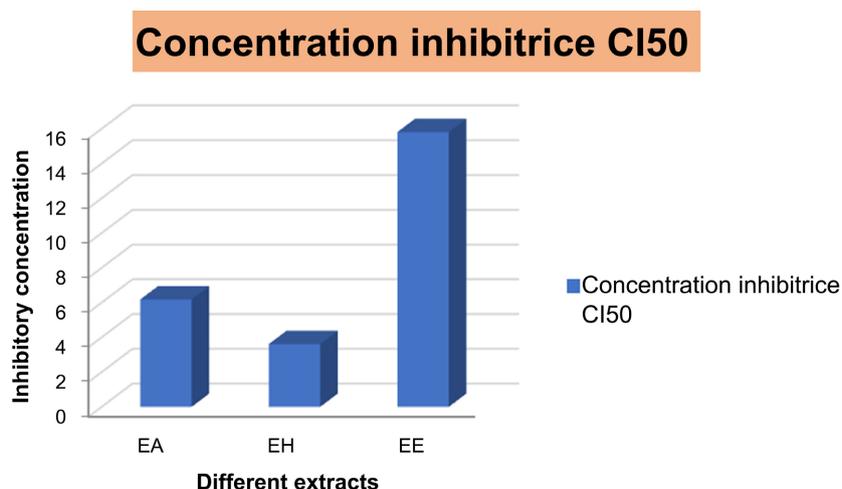


Figure 5. Evaluation of the antiradical activity in the different extracts.

5. Conclusion

This study showed that the hydro-ethanolic extract of the plant of *P. aquilinum*

presented a great wealth in polyphenols and total flavonoids. It also revealed that the other aqueous and ethanolic extracts are rich in polyphenols and less in total flavonoids. In addition, the ethanolic extract is potentially rich in anti-radical compounds. The two others (aqueous and hydro-ethanolic) are less rich in anti-radical compounds. These results allow encouraging the users and consumers of the plant of *P. aquilinum*, which contains polyphenols and total flavonoïdes in sufficient quantity. This plant, which is already consumed by the Brazzaville and Congolese population, must be better popularized, especially its use as a potential additive in place of synthetic compounds.

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Conflicts of Interest

No conflict on this article.

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Nomenclature

EGA: Gallic Acid Equivalent;

ECa: Catechin Equivalent;

Qr: Quercetin;

Rut: Rutin;

Ac.Caf: Caffeic Acid;

Ac.Chl: Chlorogenic Acid;

M_s: Dry Matter;

mg: Milligramme;

mL: Milliliter;

P. aquilinum: *Pteridium aquilinum*;

CCM: Thin Layer Chromatography;

IC₅₀: 50% Inhibitory Concentrations;

DPPH: 1,1-Diphenyl-2-Picrylhydrazyl;

TVF: Total Flavonoid Content;

TPP: Total PolyPhenol Content;

PEG400: Polyéthylèneglycole 400.