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Determination of the Chemical Composition and Evaluation of the Antioxidant Properties of the Leaves of Piper Brachyrhachis (C.H. Wright)

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ABSTRACT

Piper Brachyrhachis is a plant used in traditional medicine in Africa. Despite the biological and therapeutic virtues of this plant, no information on the content of total phenols and on the antioxidant activity exists. This study evaluates the content of total phenols, the antioxidant activity of the extracts of the leaves of this plant. The antioxidant activity was evaluated by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Total polyphenol content (TPC), total flavonoid content (TFC) and total tannin content (TTC) were evaluated using a UV spectrophotometer. The values of TPC, TFC and TTC were 34.6 ± 0.02 mg GAE/ml, 56.6 ± 0.4 mg CE/ml and 34.78 ± 0.03 mg CE/ml, respectively. The different extracts showed an average activity of trapping two free radicals of the order of IC50: 15.6 ± 0.012 µg/ml, equivalent to a %IP of 84.46 ± 1.78. The two major compounds identified and quantified by LC/MS are quercetin and ellagic acid, with a respective retention time of 15.42 min for ellagic acid (2.5 ± 0.3 µg/ml) and 26.90 min for quercetin (6.8 ± 0.2 µg/ml). The correlation between the AOA (antioxidant activity) and the TPC, TTC, TFC was with r2 = 0.999).

GAE: Gallic acid equivalent; CE: catechin equivalent; Ic: inhition concentration; IP: Inhibition percentage

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KEYWORDS:

Piper Brachyrhachis; polyphenols; antioxidant propreties; quercetine; ellagic acid; HPLC-MS

1. INTRODUCTION

Medicinal plants are important sources of biomolecules, compounds used in various fields of life, biotechnology, pharmaceuticals, cosmetics, chemistry... Plants are an important source of compounds with various biological activities (antioxidants, antimicrobials, anti-inflammatories, etc.). Several researchers, chemists, pharmacists, biologists, biochemists, doctors… are looking for new molecules with different biological functions that can respond to certain health problems, alleviate the suffering of patients or diseases that have no treatment, find solutions (new molecules) more effective for those who are resistant to existing treatments.

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We know that since ancient times, man uses plants for his daily life (to heal, to feed). In developing countries, traditional medicine is the first resort for more than half of the population, due to its geographical, economic and cultural accessibility.

Today, at least more than 70% of the people on this planet use plant extracts to treat themselves, so it has been proven that at least 70% of existing medicines are of natural origin (plants, fungi, algae, etc.) Traditional medicine is the oldest in the world, it is a frame of reference for the development of new treatments. The primary concern of any medical research is the well-being of the patient. This research concerns not only the efficiency and safety aspect, but also the requirements for the safety (safety) of new molecules in the face of different side effects presented by molecules or compounds of organic syntheses. Clinical studies have shown that herbal drugs are better tolerated by patients than synthetic derivatives (Silveira et al., 2019).

The emergence of new diseases, the resistance of certain treatments or molecules to certain diseases, remains the major concern of medical personnel. It is important to have new treatments for resistant molecules that are more effective and better tolerated by the body. Faced with the side effects of synthetic molecules, which are often harmful to the body and difficult to bear for patients, herbal medicine becomes the alternative solution. Plants and plant products are increasingly being used in therapies for various diseases (Es-safi et al., 2020).

Several articles have been published on the pharmacological properties of medicinal plants or components isolated for their properties such as antioxidant, antidiabetic, antibacterial, antiviral and antiulcerative activities (Ali Zaiter et al., 2016). For example, the antimicrobial properties of plants have been studied by researchers around the world and biological evaluation of plant extracts is essential to ensure their effectiveness.

All parts of the plants (leaves, roots, fruits, bark...., etc.) contain a wide variety of molecules having various biological properties, this is the case of antioxidant or anti-radical activity of phenolic compounds (phenolic acids, flavonoids, anthocyanins and tannins) and vitamins (Muanda et al., 2011; Silveira et al., 2019; Fernanda et al., 2012).

The side effects of synthetic drugs, the search for new treatments and new molecules are pushing medical researchers to search for natural medicines. Plant products are not only used in traditional medicines, but also in many pharmaceutical products. Indeed, many efforts have been made to study the preventive role of nutraceuticals against disease (Es-safi et al., 2020; Ali Zaiter et al., 2016). This is the case of polyphenolic compounds of plants, which have preventive activities, such as anti-mutagen, anti-carcinogen and anti-aging. Indeed, polyphenols have the ability to recover reactive oxygen species (ROS), preserving the genomic stability of cells by eliminating carcinogens and interfering with the formation of DNA adducts.

These compounds are called antioxidants and can protect biological systems from potentially harmful effects (Muanda et al., 2011). Thus, when the production balance between ROS and defense systems is disturbed, oxidative stress sets in, favouring many diseases such as cancer, atherosclerosis and other cardiovascular disorders, diabetes, accelerated aging and Alzheimer's disease (Mechchate et al., 2021).

Also as part of the search for new molecules and treatments for certain orphan diseases, we initiated this study on Piper Brachyrhachis, a plant of tropical regions, found in sub-Saharan Africa, Congo, Zambia, Tanzania..., its leaves are used in traditional medicine for its sedative and anxiolytic effects. But to our knowledge, but to our knowledge there is no scientific study in the literature that could justify its use. a methanoic extract was prepared using the method previously used by Muanda et al. (2011). a chemical screning was carried out beforehand on the extracts of these leaves, in order to determine the presence of certain chemical groups likely to have biological properties (tannins, terpenes, saponins, flavonoids, polyphenols, alkaloids, anthocyanins, reducing sugars). The objectives of this study are therefore to evaluate the various chemical compounds present in the leaves of Piper Brachyrhachis, (total polyphenols, total flavonoids, tannins), evaluate the antioxidant properties of these extracts and establish a link between the presence of these compounds and their antioxidant activity.

2. MATERIALS AND METHODS

2.1. Apparatus

-1. LC-ESI/MS analyses were performed on a LC-MS 2020 system (Shimadzu, Tokyo, Japan) coupled with an electrospray ionization source (ESI). Separation was performed on a Gemini 3 Eım C18 130 Å reversed phase column (Phenomenex, Torrance, CA, USA) of 150 mm length and 4.6 mm i.d..

-2. UV–visible analyses

UV–visible spectrophotometric analyses were carried out with an UV–visible spectrophotometer Analytikjena Specord 205 (Konrad-Zuse-Strasse 1 Jena, DE 07745).

2.2. Plants materials

Fig.1. Piper capense var. brachyrhachis C.H. Wright Verdc

Fresh leaves of *Piper Brachyrhachis* plants were collected in 2021 from Kisantu (RD Congo). The leaves were identified by Mister Landu (Laboratory of Botanic/ Faculty of Sciences University of Kinshasa). All leaves materials were dried at room temperature and were ground and sifted in a sieve (0.75 µm).

2.3. Chemicals

Chemicals Folin-Ciocalteu phenol reagent, gallic acid, sodium chloride, ethyl acetate, acetic acid, Folin-Cio calteu's phenol reagent, aluminum chloride catechin, gallic acid, p-coumaric acid, coumarin, rutin, chlorogenic acid, vitamin acid, delphinidin, orietin, ellagic acid, l-cyanidin, ellagic acid,

l-cyanidin were purchased from Across Organics. Sodium carbonate, sodium nitrite, chlorhydric acid, ethyl acetate, soduim sulfate anhydrous, ammonium phosphate, ferric ammonium sulfate, acetoninitrile, DPPH (2,2-diphenyl-1picrylhydrazyl) were obtained from Sigma and Roth (France). The chemicals used were all of analytical grade.

2.4. Samples preparations

1. Phenolics compounds extraction for LC-ESI/MS analysis & UV–visible analyses

Extraction of polyphenols from *Piper Brachyrhachis* (L.) leaves were carried out according the method of Muanda et al. (2010) with some modifications. 2 g of powder of dry leaves were macerated during 24 h under stirring at 200 rpm in 10 mL methanol–water (70– 30% (v/v)). Then, the methanol–water extract was centrifuged at 6000 rpm for 20 min, the supernatant layer was filtered, brought to 10 mL by adding methanol–water 70– 30% (v/v), and stored at 4 °C until analysis in a sealed dark brown vials.

2. *LC-ESI/MS analyses*

-Analytical conditions

The column oven was fixed at 30°C. The mobile phase was consisted by a mixture of (0.5% of formic acid in water (A) and of acetonitrile (B). The injected volume was 20µl and the flow rate was fixed at 0.5 ml/min, the employed gradient was as follows (Table 1).

Table 1: gradient of LC-ESI analyses

	Time (mn)	Pomps (B/A)	% (Elution)
1	5	B/A	10/90
2	20	B/A	25/75
3	23	B/A	25/75
4	27	B/A	90/10
5	30	B/A	90/10
6	33	B/A	10/90
7	34	Stop control (re-equilibration step)	10/90

Compounds were quantified with an external calibration method. Before the analysis, the plant extracts were filtered and diluted ten times twice.

Data were expressed in milligrams of corresponding standards per gram of dry matter. The ESI source was operated in negative mode. The nebulization gas flow was set at 1.5 L/min, the heat block temperature was fixed at 350 °C, and the desolvation line (DL) temperature at 250 °C. The probe voltage was set at −4500 V.

-Qualitative analyzes

The prepared extracts were analyzed on the LC/MS, the major peak reveals a m/z rapport [M-H] equal to 301 corresponding both to Ellagic Ac. and to Quercetin. To remove this ambiguity, the comparison of the retention times 15.4 mn and 26.9 mn of the pure standards of ellagic acid and of quercetin respectively, were carried out under the same analysis conditions.

-Quantitative analyzes

Calibration method

Quantification of standards was performed using the TIC (Total Ion Chromatogram) in order to improve detection sensitivity in comparison with the full scan mode. The method sensitivity and linearity were assessed by determining the limits of detection (LOD) and quantification (LOQ), defined as the concentrations leading to a signal-to-noise ratio (S/N) of 3 and 10, respectively. The linear calibrations curves of quercetin and of ellagic acid were established in a range comprised between 1µg/ml to10µg/ml.

3. UV–visible spectrophotometric analyses

-Determination of the Total Phenolic Content

Total phenolic contents (TPC) were measured with the method reported by Muanda et al. (2010). 100 µL of samples were added to test tubes containing 3.16 mL of distilled water followed by addition of 900 µL Folin-Ciocalteu reagent (1 N) and 200 µL sodium carbonate (Na_2CO_3, 20%). Samples were thoroughly mixed and vortexed. After 40 min incubation at room temperature, the absorbance was measured at 725 nm. For calibration curves, diluted solutions of gallic acid (1, 1.5, 2, 2.5, and 3 mg/mL) were used and total phenolic content was expressed in terms of equivalent amounts of gallic acid per gram of dry matter (GAE/g DM). All experiments were triplicated.

-Determination of the total flavonoids content

Total Flavonoids Contents were measured according to a colorimetric assay reported by Muanda et al. (2010), 20µl of samples were added to 1 ml volumetric flask containing 1 ml of distillate water. At the initial time (t0), 75 µl on $NaNO_2$ (5%) was added to the flask. After 5 minutes, 75 µl of $AlCl_3$ (10%) was added and 6 minutes after, 500 µl of $NaNO_2$ (1N) was added to the mixture. Immediately, the solution was diluted by adding 2.5 ml of distillate water and mixed thoroughly. Absorbance of the mixture, pink in color was determined at 510 nm versus the prepared blank.

The amount of flavonoid was calculated by using the standard curve of catechin was expressed as mg Catechin Equivalents/g dry matter.

-The tannins compounds

Condensed tannin content was estimated using the method described by Villarreal-Lozoya et al. (2007) with some modifications. An aliquot of 0.5 g of powered obtained after lixiviation (n-hexane) was placed in a centrifuge tube and 15 ml of 1% HCL in methanol was added to each sample. Each tube was vortexed and placed in water bath at 35°C with constant shaking for 20 minutes and vortexing every 5 minutes. After incubation, the tubes were centrifuged (1536xg) and the supernatants were extracted. Aliquots of the supernatants (100µl) were placed in two separate assay tubes,

one for the sample determination and the other for blank determination. Samples and blank were incubated for exactly 20 minutes after adding 5 ml of vanillin reagent (0.5 g of reagent and 200 ml of 4% HCl –methanol) to samples and 4% HCl in methanol to the blanks. After 20 minutes, absorbance was read at 500 nm from each sample and blank using UV spectrophotometer. Samples absorbance was rectified with the blank standard and compared against a standard curve made with catechin. Results were express as mg catechin equivalent (CE) of lixiviating sample. Analyzes were triplicate.

-Antioxidant activity

DPPH tests

The DPPH radical scavenging activity was evaluated according to the method described by (Sharififar et al. (2009); Kedare et al. (2011), slightly modified. 2.9 mL of 100 µM DPPH solution in methanol was mixed with 1 mL plant extract. The reaction mixture was incubated *in* the dark for 30 min and the optical density was recorded at 517 nm against the blank. For the control, 100 µl of DPPH solution in methanol was mixed with 2.9 mL methanol and optical density of solution was recorded after 30 min. The DPPH radical scavenging activity was expressed in terms of IP% and Ic50.

IP% = (A_0 - At)/A_0, *IP : inhibition percentage, A_0: absorbance of DPPH solution without sample at zero time, A_t : absorbance of DPPH solution with the sample at 30 minutes. I_C50 (Half percentage inhibition concentration)*

2.5. Statistical analyses

For all experiments, the average value and standard deviation from three replicates were calculated. Statistical analysis (ANOVA) was conducted using SAS statistical software (SAS Institute, NC) with $p < 0.05$ as significance level.

3. RESULTS AND DISCUSSION

1. LC –ESI/MS analyzes

-Identification of major bioactive compounds in Piper Brachyrhachis leaves

-Qualitative analyzes

The two major compounds in methanol/water leaves extracts of Piper Brachyrahchis were identified and quantified by LC-ESI/MS. The LC-ESI/MS analyses of some polyphenol standards allowed us to determine the retention times and the m/z ratios of Ellagic acid and Quercetin as being the two major compounds (fig.2).

-Quantitative analyzes

Method sensitivity was assessed by determining the limits of detection (LOD) and quantification (LOQ), defined as the concentrations leading to signal-to-noise (S/N) values of 3 and 10, respectively.

For quantitative analyzes, we have determined the calibration curves for ellagic acid and for quercetin; $R^2 = 0.998$ and 0.988 respectively. From these calibration curves, leaves extracts of *Piper B* contain 1.26 ± 0.15 µg/g DM of Ellagic Acid and 3.46 ± 0.11 µg/g DM of Quercetin.

The concentrations of quercetin in the. leaves extracts were calculated from the calibration curve using the mean response values of three sequential injections. Thus the *Piper Brachyrhachis* leaves extracts contained 6.8 ± 0.2 µg/ml of quercetin which corresponds to 3.46 ± 0.11 µg g-1 dried *Piper Brachyrhachis material*.

The results of these analyses show that, quercetin content in the *Piper.Brachyrhachis* extracts were higher than ellagic acid.

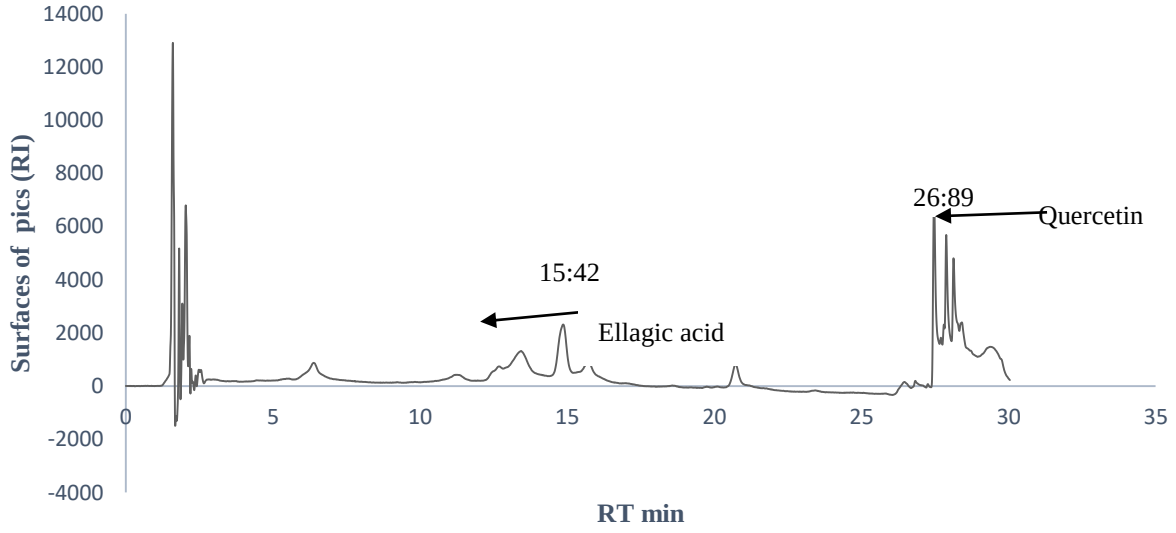

Fig 2.a Chromatogram profile of extract

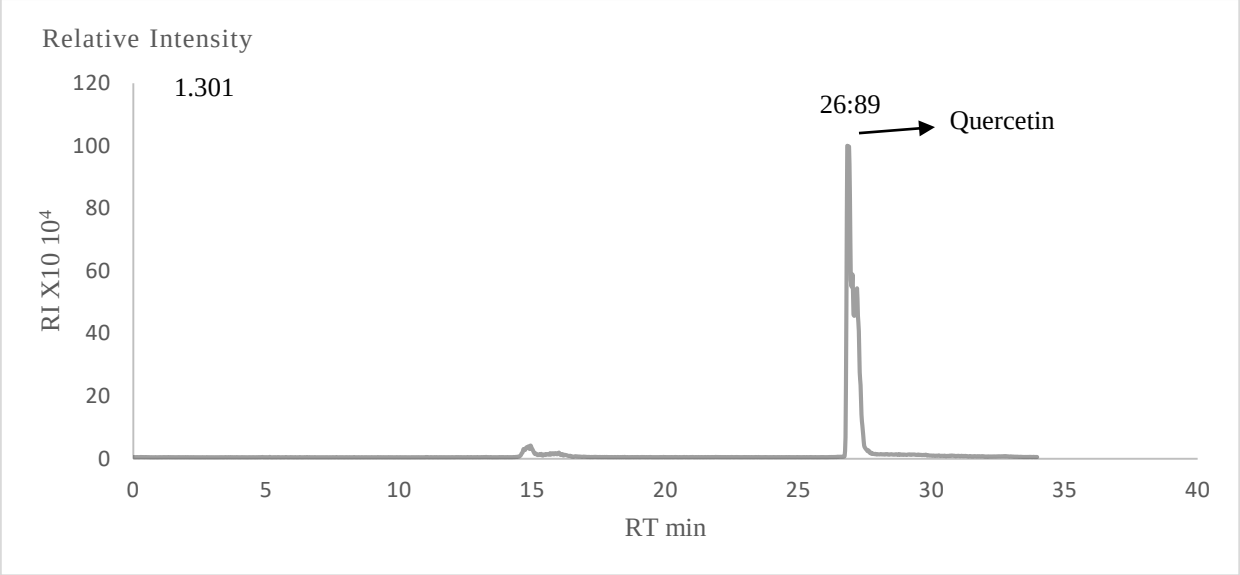

Fig.2b. Chromatogram of Quercetin M/Z: 301

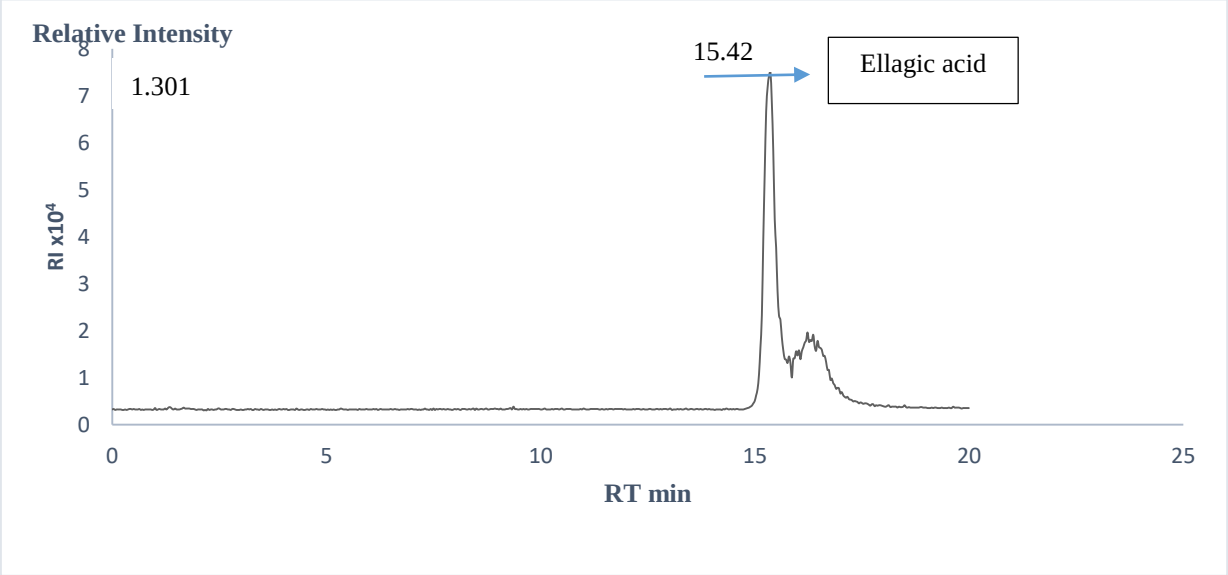

Fig.2c. Chromatogram of Ellagic acid (M/Z: 301) RT: retention time, min: minute

2. UV- spectrophotometer analyzes

1. Total phenolic content

The folin-Ciocateu test was used to evaluate TPC (Total phenolic compounds) in *Piper* Brachyrhachis leaves (Muanda et al., 2010). The assay of total polyphenols was carried out at 725 nm against a gallic acid standard. The TPC concentration is expressed in gallic acid equivalent. The calibration curve obtained was y = 4675,8x - 0.1096, within a range 0.05 to 0.3 mgEGa/ml (total number of data points = 6)). (*mgEGa/ml: microgram equivalent gallic acid/ml*). The correlation coefficient (r^2 = 0.9945). The results of the TPC analysis, after multiplication of the dilution factors gave an average absorbance corresponding to 0.6983 ± 0.02. Which gives 34,6 ± 2 mg EGa/ml. That corresponds to 17,3 ± 0,1 mg EGa/SM. The test was repeated three times.

2. Favonoids Content

The calibration curve is made as a function of catechin. The results of the analyzes are therefore expressed in catechin equivalent. The $NaNO_2$, $AlCl_3$ and NaOH, were used to evaluate the flavonoid compounds. The calibration curve obtained was y = 1996,8x - 0.0029, within a range 3.75 to 120µg CE/ml (total number of data points = 6) (CE: Catechin equivalent). The correlation coefficient (r^2 = 0.9952).

The results of the TFC (Total flavonoid compounds analysis, after multiplication of the dilution factors gave an average absorbance corresponding to 0.3332 ± 0.02. Which gives 54,6 ± 0.4 mg CE/ ml. That corresponds to 27 ± 0,2 mg CE/SM. The test was repeated three times.

3. The tannins compounds

The calibration curve is made as a function of catechin. The results of the analyzes are therefore expressed in catechin equivalent. The calibration curve obtained was y = 0.3959x - 0.0013, within a range 2 mg EC/ml to 14 mg Ect/ml (total

number of data points = 7) (CE: catechin equivalent). The correlation coefficient (r^2 = 0.895).

The results of the tannin analysis, after multiplication of the dilution factors gave an average absorbance corresponding to 0.3332 ± 0.02. Which gives 34,78 ± 0,03 mg CE/ml. That. The test was repeated three times. The results of TPC, TFC, TTC were represented in table 2.

Table 2. Results of AOA, TPC, TFC TTC

DPPH		TPC	TFC	TTC
%IP	IC50 CVE mg/mL	mg EGa/ml	mg CE/ mL	mgCE/mL
84,46 ± 1.78	0.0156 ± 0.012	34,6 ± 0,02	54,6 ± 0.4	34,78 ± 0,03

Total phenolic compounds (TPC), Total Flavonoid compounds (TFC), Total Tannin Compounds, CVEmg/mL: mg vitamin c equivalent; milligram equivalent gallic acid; mg CE: milligram equivalent catechin

4. In vitro antioxidant activity

1,1-Diphenyl-2-pycrylhydrazyl (DPPH) is a stable nitrogen-centered free radical whose color changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore, radical scavengers (Brand-Williams et al.,1995). The scavenging effects of extracts from *Piper Brachyrhachis* tested on the DPPH radical were measured. The scavenging activity of extracts on inhibition of the DPPH radical was related to the concentration of extracts added, the activity increased as a result of increasing concentration for each species. The scavenging effect was expressed as IP% (inhibition percent) or Ic50 (half inhibition concentration) of Equivalent vit C. DPPH is method is extremely used for screening antioxidants activity. Several authors have reported that hydrolic extracts from stems, barks or leaves, contain high amounts of bioactive compounds and have showed in vitro a scavenged DPPH (Gomati et al., 2015; Zhiping He, et al., 2011). The DPPH solution decrease with *Piper Brachyrhachis* leaves extracts and the scavenging activity evaluated and express in percentage IP (%) or in Ic50. The antioxidant activity is evaluated according to vitamin C.

A calibration curve is thus established (y = -491.51x + 1.0195), r2 = 0.9931 and the results of the analyzes are expressed in vitamin C equivalent. IP (%) = 84.46 ± 1.78 and Ic50 calibration curve (Y = 2734.9 x -1.2554) r2 = 0.992, the Ic50 value calculated was (0.0156 ± 0.012) VCE mg/ml. within a range 0 mg EC/ml to 0.0025 mg Ect/ml (total number of data points = 5) (VCE: C Vitamin equivalent).

5.Correlation between antioxidant activity and phenolic compounds

There is indeed a close relationship between phenolic compounds and antioxidant activity. These are shown by the values of r^2 which are all equal to 0.999 (fig.3).

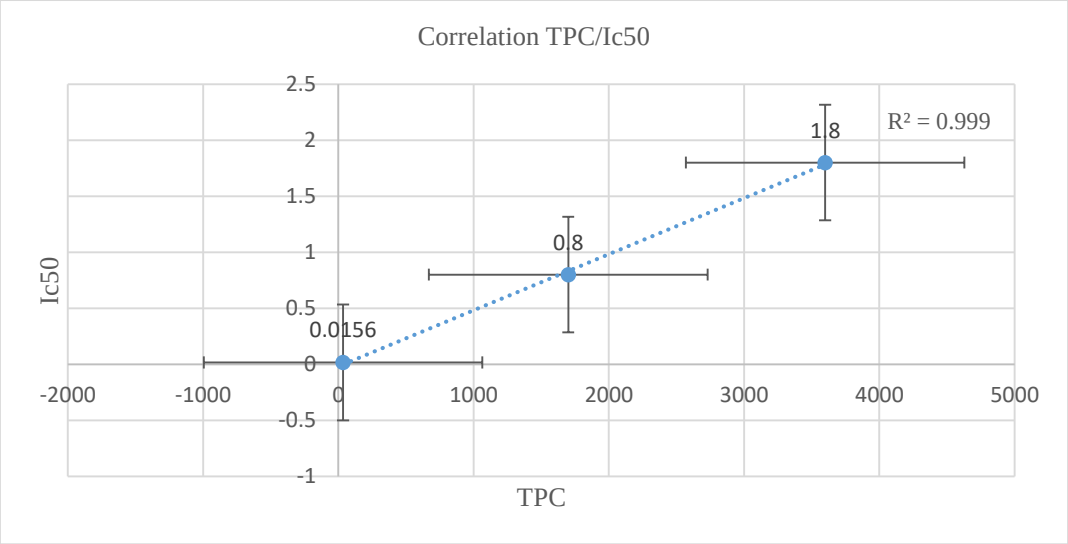

Figure 3a. Correlation lines between (TPC) and (AOA)

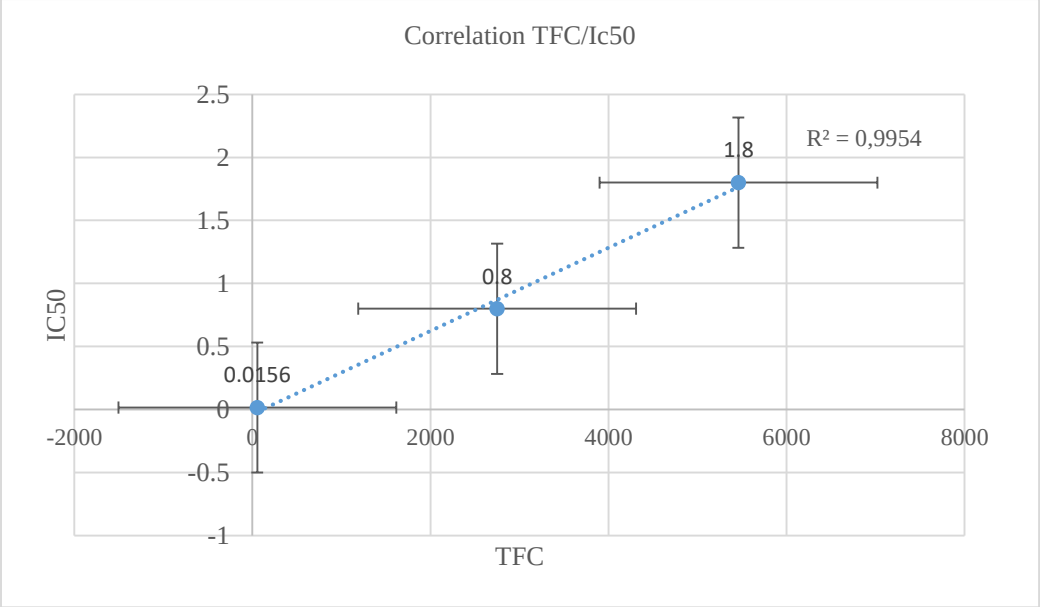

Figure 3b. Correlation lines between (TFC) and (AOA)

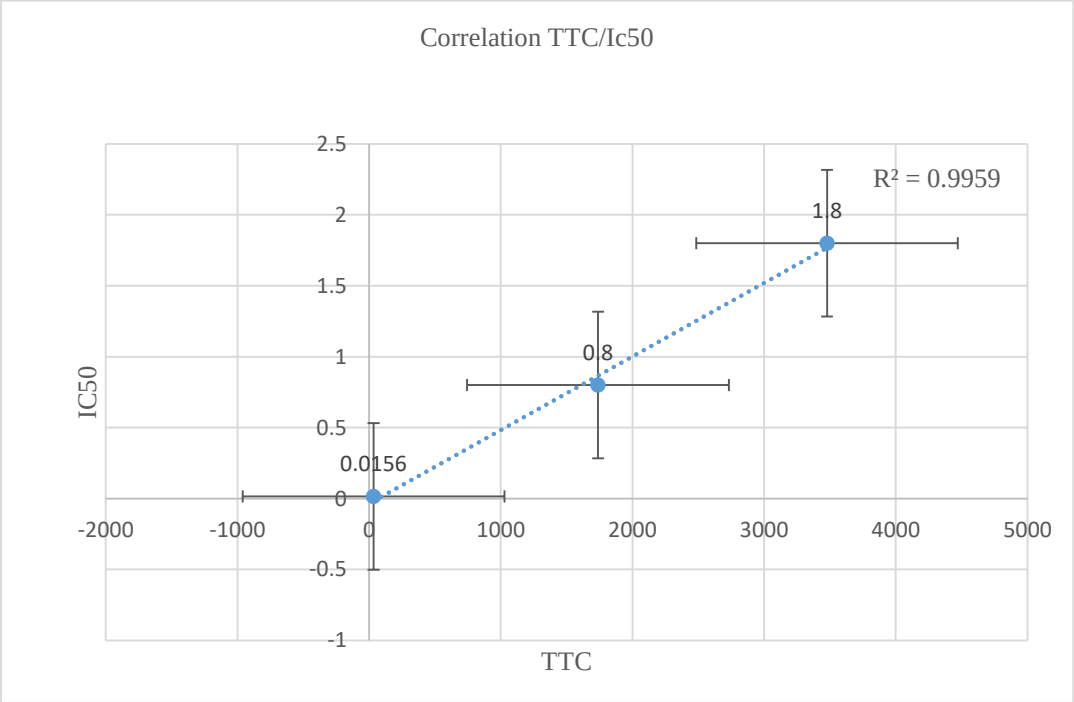

Figure 3c. Correlation lines between (TTC) and (AOA).

TPC: total phenolic compound; TFC total flavonoid compound; total tannin compound; antioxidant activity

By comparing the value of the Ic50 with those of the other leaves which were the subject of our study previously, the leaves of *Daniella oliveri* & *Vitex doniana*, we find that the value of the IC50 of *Piper Brachyrhachis* is much higher, 15.6µg/ ml (*Piper Brachyrhachis*) > 2.7 (*Daniella Oliveri*) & 2.9 µg/ml *(Vitex doniana*) (Muanda et al., 2010). Ours results compared to the extract from Mangosteen fruit Ic50: 10.94 µg/ml) our result is also higer (Werayut Pothitirat et al., 2010). These values showed that the *Piper Brachyrhachis* leaves extracts has significant antioxidant activities. The Phytochemical screening of the leaves extracts of *Piper Brachyrhachis* revealed also the presence multiple of phenolic compounds, flavonoids and tannins. The presence of that compounds reveals its activity against several diseases, like quercetin and allegic acid.

Quercetin (Figure 4) is found in abundance in leafy vegetables, citrus fruits and berries. The presence of five hydroxyl groups in its structure gives it properties high antioxidants (Simon et al., 2019).

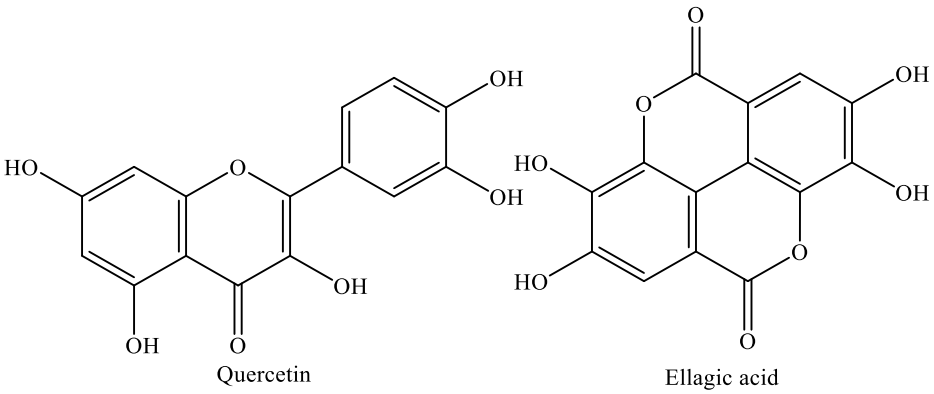

Figure 4: Chemical structure of quercetin and ellagic acid

Quercetin is an antioxidant compound, capable of neutralizing free radicals and chelated metal ions from the transition. For example, it successfully inhibits lipid and lipoprotein peroxidation (Simon et al., 2019; Sahoo et al., 2011; Karakaya et al., 1999). It can also reduce inflammation, promote collagen fiber growth and inhibit oxidative stress mediators (Ghedadba 2014). Many studies have shown that quercetin is responsible for antioxidant, anti-inflammatory, healing and anti-aging properties (Simon et al., 2019).

Research has shown that people taking a quercetin supplement experienced stabilization in systolic and diastolic blood pressure. On stress, when the body is stressed it produces cortisol and Cortisol is a hormone that damages muscle cells, it causes protein breakdown in the body, quercetin combats these effects (Debnath et al., 2021). On muscular endurance, a recent study on athletes and people starting physical activity showed that quercetin increased endurance. For example, a daily dose of 1000 milligrams of quercetin increased the number of mitochondria in muscle cells, which aids in energy production (Debnath et al., 2021; Baghel et al., 2012; Zhang, M . et al., 2011).

For ellagic acid, it is generally found in plants as hydroxybenzoic acid. However, it is mainly located in plant vacuoles as hydrolysable tannins called ellagitannins. Ellagitannins are known as glucose esters of ellagic acid which can be hydrolysed to ellagic acid.

Ellagic acid is present in high concentrations in many fruits, including strawberries, raspberries, cranberries and grapes. In vitro and in vivo studies of ellagic acid have demonstrated its pharmacological benefits. Anti-mutagenic properties, antioxidant, anti-inflammatory and anti-carcinogenic activities, as well as a better conservative effect against oxidative stress compared to vitamin E mediators were also evaluated on batteries and in some Mammals (Ghedadba 2014).

CONCLUSIONS

The aim of this study was to evaluate the antioxidant power, to determine the composition of polyphenolic compounds and to establish the relationship between the presence of these compounds and the antioxidant activities of *Piper Brachyrhachis* leaf extracts. Indeed, the literature has remained silent on the antioxidant properties and the chemical composition of this plant. No information exists on this plant although it is widely used in traditional medicine. It has been shown that extracts of piper B. leaves contain chemical compounds with antioxidant properties. Also a correlation between these chemical compounds and the antioxidant activity at well was established with a ($R^2 = 0.999$). Thus, one can well affirm that the extracts of leaves of *Piper Brachyrhachis* presenting interesting antioxidant properties, could justify their use in traditional medicine.

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