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PHYTOCHEMICAL STUDY AND EVALUATION OF ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF *VITEX DONIANA* SWEET (VERBENACEAE)

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ABSTRACT

Owing to difficulties encountered in the use of synthetic antioxidants, research is moving more and more towards natural antioxidants particularly those from plants. It is in this context that we were interested *Vitex doniana* Sweet, a plant commonly used in the Ivorian pharmacopoeia for the treatment of several diseases. After an experimental study on a phytochemical screening of the methanolic extract of the plant, a quantitative estimate of total polyphenols (by the Folin-Ciocalteu method) and total flavonoids (by the method aluminum trichloride) was performed. Then; the antioxidant activity of the extract was evaluated through three methods: the method of DPPH (2.2 diphenyl picrylhydrazyl 1), the FTC (ferric thiocyanate) method and TBARS (thiobarbituric acid) method. The results obtained are shown the presence of various compounds including polyphenols groups. Furthermore, inhibition of DPPH radical coupled to the inhibition of lipid peroxidation by test FTC and the TBARS showed significant antioxidant activity for this extracted with a percent inhibition of 53% recorded by FTC and 52% by TBARS. The antioxidant activity of the observed plant would be partly due to its abundance of polyphenols.

KEYWORDS: *Vitex doniana*, methanolic extract, phytochemical screening, antioxidant activity, Côte d'Ivoire.

INTRODUCTION

The use of medicinal plants is an ancient practice, but it has grown significantly in recent times. Several factors may explain this craze for example, the decrease in purchasing power, the high cost of conventional medication, mistrust against synthetic products and the desire to consume Bio "natural".[1,2] These factors justify the use of traditional medicine for people in developed countries to their health care.[3] To help these people to make better use, medicinal plants have been the subject of several scientific studies on the treatment of diseases.[4,5]

In Côte d'Ivoire as everywhere in Africa, many researches have focused on anti-infective properties of plants, but there is very little data on the antioxidant effects of these [6]. But these antioxidants are one of the best ways to fight against oxydantif stress appears to be the cause of serious multifactorial diseases.[7]

According to several authors, oxidative stress is involved in various diseases such as diabetes, rheumatism, Alzheimer's disease, cancer and cardiovascular disease.[8,9] Furthermore, most of these diseases caused by oxidative stress appear with age because aging decreases antioxidant defenses and increases mitochondrial propagating radicals.[10,11]

To fight against oxidative stress and its associated pathologies, synthetic antioxidants such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are regularly prescribed.[12] But in recent years, because of their negative effects observed on human health, research is increasingly oriented to the natural antioxidants including those from plants.[13] It seemed therefore interesting to include this work in this research context.

The purpose of this study is to evaluate the antioxidant properties of *Vitex doniana* Sweet. (Verbenaceae) plant used in African pharmacopoeia to treat many diseases.[14]

MATERIALS AND METHODS

1- Materials

1-1 Plant material

The plant material is made up of *Vitex donniana* Sweet trunk bark and harvested at Lataha (Korhogo), north of the Ivory Coast. The plant was identified by Professor Ake-Assi of the National Floristic Centre (NFC) of University FHB Cocody-Abidjan.

1-2 Reagents

Were used DPPH (2, 2'-diphenyl-1-picrylhydrazyl), methanol and acetic acid provided by HiMedia (India), quercetin and vitamin C or ascorbic acid from Sigma (USA) and of Folin-Ciocalteu reagent, formaldehyde and sodium acetate obtained from Merck (Germany). Methanol and acetic acid obtained from Fischer Scientific (USA) whereas the sodium carbonate is from Rankem (India).

2- METHODS

2-1- Preparation of extracts

This preparation is performed according to the method described by **Bidie et *al*.**[15] Indeed, crushed 50 g of plant bark was mixed with 1.5 L of methanol 96%. The resulting mixture was stirred for 48 hours at room temperature (25°C) using a magnetic stirrer type IKAMAG RCT (Staufen, Germany). Then, the mixture is filtered three times on cotton and on Whatman filter paper 3 mm. The filtrate was evaporated at reduced pressure and 40°C using a rotary evaporator Buchi 461 Watter Batch (Strasbourg, France). The resulting powder was used to make the different tests.

2-2- Phytochemical screening

Phytochemical tests for tannins, polyphenols, flavonoids, alkaloids, sterols, polyterpenes, saponins and carotenoids were conducted according to the methods described by **Ouattara et *al***[2] and **Yeo et *al*.** [16]. with minor modifications.

2-2-1- Test for alkaloids (reactions Dragendorff and Bouchardat)

Six milliliters of plant extract were evaporated. The residue was taken up in six milliliters of alcohol at 60° and the alcoholic solution thus obtained was divided into two test tubes. In the first tube was added two drops of Dragendorff reagent. The appearance of a precipitate or an orange color indicated the presence of alkaloids. In the second tube was added two drops of reagent Bouchardat. The appearance of a reddish-brown color indicated a positive reaction to the presence of alkaloids.

2-2-2- Test for tannins

About 0.5 g of the extract was boiled in 10 mL of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

2-2-3- Test for polyphenols

To 2 mL of plant extract was added a drop of alcohol solution of ferric chloride at 2%. The appearance of a dark green or lighter or darker blue color indicated the presence of polyphenolic derivatives.

2-2-4- Test for sterols and polyterpenes (reaction Liebermann)

After evaporation to dryness 5mL of each solution in a capsule on a sand bath without charring, the residue was dissolved in hot acetic anhydride and 1 mL in a test tube, we poured cautiously with 0.5 mL of concentrated sulfuric acid along the tube wall to the solution. The applications to the interphase of a purple or purple ring, turning blue to green, indicate a positive reaction.

2-2-5- Test for flavonoids

Three methods were used to test for flavonoids. First, dilute ammonia (5 mL) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 mL) was added. A yellow colouration that disappear on standing indicates the presence of flavonoids. Second, a few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow colouration indicates the presence of flavonoids. Third, a portion of the extract was heated with 10 mL of ethyl acetate over a steam bath for 3 minutes. The mixture was filtered, and 4 mL of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration indicates the presence of flavonoids.

2-2-2-6- Test for saponosides

A volume of two milliliters of each extract was evaporated and taken up in five milliliters of water. After vigorous stirring, the foaming of more than one centimeter, stable and persistent high for 30 minutes indicated the presence of saponins.

2-2-2-7- Test for cardiac glycosides (Keller-Killiani test)

To 0.5g of extract diluted to 5 mL in water was added 2 mL of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

2-2-2-8- Test for reducing compunds

Detection of reducing compounds was performed using the Fehling test. Fehling's solution is a mixture of two solutions A (1.73 g CuSO4 in 25 mL water) and B (8.5 g of tartrate of potassium and sodium plus 2.5 g NaOH in 25 mL equal volumes of distilled water. In a test tube containing 5 mL of extract, are added 5 mL of Fehling's solution. The formation of a precipitate brick red after 3 minutes of heating in a water bath at 70°C indicates a positive reaction.

3- *IN VITRO* EVALUATION OF ANTIOXIDANT ACTIVITY OF PLANTS

3-1- determination of the polyphenols

For a better determination of the level of phenolic compounds in each extract, three repetitions were applied, and standard deviation was calculated.

3-1-1- Determination of total phenols

The total phenolic contents of four extracts were determined by the Folin-Ciocalteu method.[17,18] To 0.5 mL of each plant extract of concentration 0.1 mg/mL, were added respectively 5 mL of Folin-ciocalteu diluted 1/10 in distilled water and 4 mL of sodium carbonate (1M). The whole is incubated at room temperature for 15 minutes. The optical densities (OD) are then read in a spectrophotometer at 765 nm against a blank. Gallic acid was used as standard and prepared under the same conditions as above with a solvent mixture of methanol/water (50:50, V/V) at concentrations ranging from 0 to 0.5 mg/mL. The total phenolic contents of the extracts are expressed in milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

3-1-2- Determination of total flavonoids

The technique used for the determination of the levels of total flavonoids extracted from ours plants the colorimetric method of aluminum trichloride described by **Chang *et al.*[19]** modified by **Yi et *al.*,[20]** Thus, 0.1 mL of 5 mg/mL of each extract are collected, to which are successively added 1.5 mL of methanol, 0.1 mL of 10% aluminum trichloride, 0.1 mL of potassium acetate (1M) and 2.5 mL of distilled water. After incubation at room temperature for 30 minutes, the optical densities were measured in a spectrophotometer at 415 nm. A methanolic solution of quercetin with concentrations ranging from 0 to 100 mg/mL is used as a standard. The contents of flavonoids extracts are expressed in milligrams of quercetin equivalent per gram of extract (mg QE/g of extract).

3-1-3- Determination of total flavonols

Determination of total flavonols plants Concentrations of total flavonols plant extracts were determined according to the method of **Kumaran and Karumakaran.[21]** To 2 mL extract (0.1 mg/mL) were added 2 mL of an ethanolic solution of aluminum chloride and 3 mL of 2% sodium acetate (50 g/L). After 2 hours 30 minutes of incubation at 30°C, the absorbance is measured spectrophotometrically at 440 nm. Quercetin prepared at concentrations ranging from 0 to 100 ug/mL was used as standard. The levels of total flavonols extracts are expressed in quercetin equivalents (mg QE/g of extract).

3-2- Measurement of anti-radical power

The measurement of the antiradical activity of plant extracts was performed by testing the 2, 2- diphenyl-1-picrylhydrazyl (DPPH) according to the method of **Parejo et *al.*[22]** From a stock solution of each plant extract to 0.1 mg/mL, a concentration range is prepared by successive doubling dilution of 1.56 mg/mL to 100 mg/mL. Then, each extract concentration, the same volume of a methanolic solution of DPPH is added. After 30 minutes of incubation at room temperature (37°C) and protected from light, the absorbance is read in a spectrophotometer at 517 nm against a blank sample (0 mg/mL of extract). Vitamin C (100 mg/mL) which is the reference material is prepared in the same conditions. The percentage inhibition of DPPH radicals is calculated by the following formula:

Inhibition (%) = [(Abs blank - Abs sample / Abs blank)] x 100.

Abs Blank is the absorbance of the control reaction (containing all reagents except the test compound) and Abs sample, the absorbance of the test compound.

From a curve representing the percentage of inhibition of DPPH radicals versus concentrations of the extracts and vitamin C, concentrations of extract and vitamin C that reduces 50% of DPPH radicals (IC_{50}) are determined and compared.

3-3- MEASUREMENT OF INHIBITION OF LIPID PEROXIDATION OF PLANTS

3-3-1- By FTC (ferric thiocyanate) method

This method measures the level of peroxides formed by measuring the complex formed by ferrous ion.[23]. Thus, in protected foil bottles are separately dissolved 4 mg of plant extract in 4 ml of ethanol (99.5%). In these mixtures, were added 4.1 mL of linoleic acid (2.5% in ethanol at 99.5%), 8.0 mL of phosphate buffer (20 mM, pH 7.0) and 3.9 mL of distilled water to a final volume of 20 mL. Quercetin (reference molecule), prepared under the same conditions is used as a positive control. The bottles containing mixtures were incubated in a water bath at 45°C. During incubation, every day (24 h), 0.1 mL of the mixture is collected in test tubes. To this amount are successively added 9.7 mL of ethanol (75%), 0.1 mL of ammonium thiocyanate (30% in distilled water) and 0.1 mL of iron II chloride ($FeCl_2$) to 20 mM of hydrochloric acid (HCl 3.5%). After 3 minutes of incubation at room temperature, the absorbance of the resultant red color is measured using a spectrophotometer at 500 nm. The assays are performed for 7 days until the absorbance of the control reaches its maximum value.

3-3-2- For TBARS (thiobarbituric acid) test

This test is used to evaluate the inhibitory potency of the plant extract by the assay of the complex formed between the thiobarbituric acid and one of the decomposition products of peroxides formed (malondialdehyde) during lipid peroxidation. The assay is the last day (7th) of the FTC test. Thus, to 2 ml of the mixture, were added 1 mL of an aqueous solution of trichloroacetic acid (20%) and 2 mL of an aqueous solution of thiobarbituric acid (0.67%). The mixture is then incubated in a boiling water bath (100°C). After incubation for 10 minutes followed by centrifugation at 3000 rev / min for 10 minutes, the supernatant is

recovered for reading on a spectrophotometer at 532 nm against a blank containing ethanol instead of the mixture. The percentages of inhibition of lipid peroxidation were determined according to the following formula:

Inhibition (%) = 100 - [(Abs sample / Abs blank) x 100]

Abs blank is the absorbance of the blank. (No excerpt) and Abs sample, the absorbance of the extracts of the plants and quercetin.

4- STATISTICAL ANALYSIS

The statistical analysis was performed using one-way analysis of variance (ANOVA) of the multiple test of comparison of Tukey-Kramer. The level of significance was determined in comparison with the control group. $p<0.05$ was considered significant. All values are expressed as mean ± SEM.

RESULTS AND DISCUSSION

Extraction efficiency

The extraction yield was 8.5%. The extract obtained was characterized by the appearance, color and performance. These are presented in **Table 1**.

Table 1: Mass, yield, Appearance and color of extracts obtained.

Mass (g)	Appearance	Color	Yield (%) relative to the weight of the ground material
100	Powder	Red-Brown	8.5

Phytochemical screening

The phytochemical screening performed on the tested extract gave the results summarized in **Table 2**. The analysis of this result reveals the presence of all chemical groups we sought in this study namely polyphenols, tannins, flavonoids, reducing compounds, cardiac glycosides and alkaloids. Only the absence of saponins in the extract was found. Furthermore, based on the intensity of the color observed in the qualitative tests, we can say that our extract is rich in polyphenols, alkaloids and reducing sugars. Flavonoids and tannins that are part of phenolic compounds[24] are, however, significant proportions compared to sterols and polyterpenes.

Table 2: Various groups of compounds highlighted in the extract.

Polyphénols		+++
Flavonoids		++
Alkaloides	Dragendorff	+
	Mayer	+++
Tannins	Catechic	++
	gallic	-
Sterols and polyterpenes		+
Reducing Compounds		+++
Cardiac glycosides		++
Saponins		-

Key: (-) : Absent ; (+) : Present in low concentration ; (++) : Present in moderate concentration ; (+++) : Present in high concentration.

Determination of phenolic compounds

The results of the quantitative determination of total phenols, flavonoids and our extract flavonols are summarized in **Table 3**. The levels determined are 72.79 ± 3.04 mg GAE/g of extract for total phenols, 38.35 ± 1.69 mg QE/g of extract for total flavonoids and 20.12 ± 2.70 mg QE/g of extract for flavonols.

Tableau 3: Levels of phenolic compounds of extract tested.

Methanolic Extract of burk of *Vitex doniana*		
Total phenols (mg GAE/g of extract)	Total Flavonoids (mg QE/g of extract)	Total Flavanols (mg QE/g of extract)
72.79 ± 3.04	38.35 ± 1.69	20.12 ± 2.70

Similar results have been reported about the green tea, fruit and grape juice.[25,26] If we compare our results with those obtained with the dates considered rich in phenolic compounds (5720-6610 mg / g).[27] and those of grape seed (7500-40400 mg / g).[28], we can say that our extract is rich in phenolic substances. However, it has been reported through several works that the content of phenolic compounds in plants would be strongly

influenced by a number of intrinsic and extrinsic factors such as the degree of maturation of the plant, the period of storage, the climate and the nature of the ground.[29,30,31]

Indeed, plyphénols are secondary metabolites synthesized by the plants during their development.[32,33] They are classified into several groups including those identified in our sample are phenols, flavonoids and tannins.[34]. it should be noted that the main reason flavonoids were selected to be assayed, is that they are the most important polyphenol class, with more than 5,000 compounds already described.[35]. In addition, they are present almost everywhere in plants.[36] and can be recognized as pigments responsible for the therapeutic properties of these.[37]

Antioxidant power

Figure 1 presents the results of the anti-radical activity of the tested extract. For all concentrations, we observe that this extract has antioxidant activity lower than that of quercetin. However, it showed a higher antiradical potential than that obtained on Mallotus oppositifolius (19%) by **Asante *et al.*[38]**. Moreover, we note that the results of antioxidant activity recorded by the FTC test (53%) and that obtained by TBARS (52%) are fairly similar. (**Figures 2 and 3**).

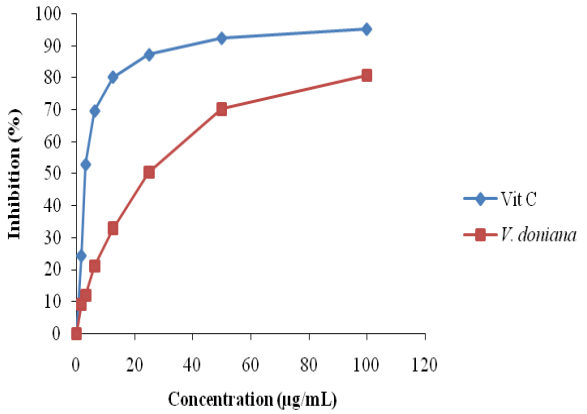

Figure 1: Anti-radical activity of *Pericopsis laxiflora* extract and vitamin C.

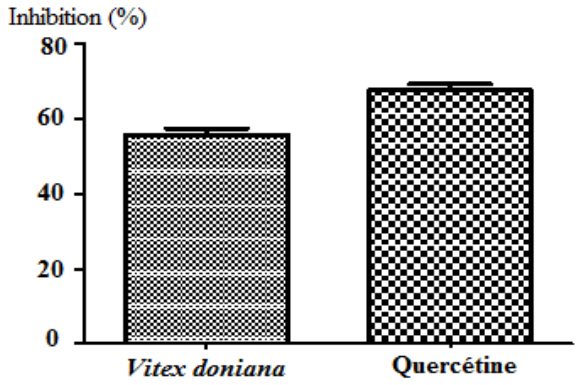

Figure 2: Antioxidant activity of *Vitex doniana* extract and quercetin by FTC method.

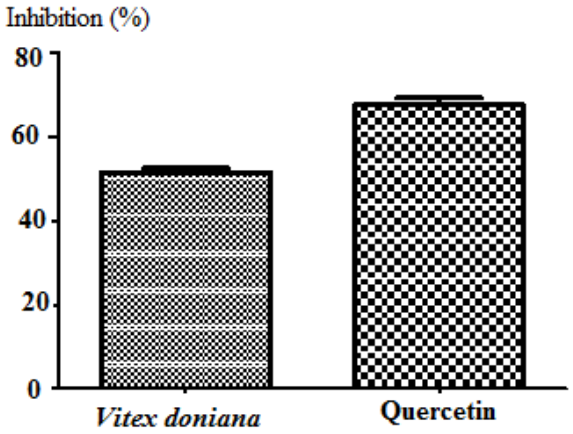

Figure 3: Antioxidant activity of *Vitex doniana* extract and quercetin by TBARS method.

Comparable results were obtained by other authors who have shown that there is an excellent correlation between total phenolic profile and antioxidant activity of plant extracts (r = 0.92) suggesting that phenolic compounds are responsible for the antioxidant activity of these extracts.[39,40]

Indeed, these phenolic compounds are synthesized by plants as a response to stress conditions such as infections, injuries and UV radiation.[41] As antioxidants, they can interact in a synergistic or antagonistic way to create an effective system vis-à-vis the free.[42] In additionthrough their ability to scavenge reactive oxygen species (ROS), antioxidants can inhibit the oxidation process of cholesterol and therefore decrease the risk of developing diseases associated with oxidative stress including cancer, cellular aging and cardiovascular disease.[43,44] This could justify the benefit of using more natural antioxidants as bioactive components in our food.

CONCLUSION

In the present work, it was shown that phenolic compounds in the extract tested are responsible for the observed antioxidant activity. *Vitex doniana* could therefore be used to prevent oxidation of cells to fight against cellular aging. Also, it would be essential in the prevention and treatment of other diseases associated with oxidative stress such as cancer, inflammatory and cardiovascular diseases. Moreover, these results could contribute to the development of natural antioxidants as well as in the pharmaceutical cosmetics and food.

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