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Phytochemical Study and Evaluation of Antioxidant Power of Selective Tannic Extracts of Three Medicinal Plants of Ivorian Flora

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ABSTRACT

This work is devoted to phytochemical study of tannic extracts from the leaves of *Combretum paniculatum* (CP), *Mallotus oppositifolius* (MO) and *Ximenia americana* (XA), three medicinal plants of Ivorian flora, used in traditional medicine against certain types of cancer. Qualitative analysis by phytochemical screening of these extracts, performed by means of TLC, revealed in addition to hydrolysable and condensed tannins, the presence of phenolic acids and flavonoids. Total tannin contents determined by permanganometry and spectrophotometry are respectively 41.03 ± 0.77% and 12.3648 ± 0.0004% for MO; 20.03 ± 0.44% and 11.7974 ± 0.0001% for XA; 18.76 ± 0.89% and 11.6026 ± 0.0008% for CP. Condensed tannin content in XA leaves (234.135 ± 0.003 µg ECT/mg) is considerably higher than in CP (37.731 ± 0.001 µg ECT/mg) and MO (19.082 ± 0.001 µg ECT/mg). All XA extracts (aqueous and ethyl acetate) have shown good antioxidant activity with CR_{50}s which are lower than that of vitamin C (reference antioxidant). A correlative relationship was found between tannin content and antioxidant activity.

Keywords: medicinal plants, tannins, antioxidant activity, DPPH, Côte d'Ivoire

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INTRODUCTION

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The state of environment, excessive consumption of synthetic drugs and food additives contribute significantly to the excessive formation of reactive oxygen species (ROS). Nowadays, it is proven that ROS, very reactive substances, are involved in etiology of much pathology. The concentration of ROS is maintained at reasonable doses by reducing them with antioxidant agents, which have become increasingly interesting to research in recent years[1]. For this reason, special attention is focused on plant extracts containing antioxidant molecules, including tannins and other phytophenols. Tannins are vegetable polymers of polyphenolic compounds with high molecular weight (500 to 3000 Da). These secondary metabolites have capacity to bind's protein substances. They differ in their chemical structure and are traditionally classified in three groups: hydrolysable or true tannins (esters of gallic acid and its derivatives with sugars), condensed tannins (condensation products of catechins, leucoanthocyanidins and hydroxystilbenes) and complex tannins (molecules containing both a catechin (or flavan-3-ol) unit linked to a gallotanin and ellagitanin unit) [2,3]. In addition to their proven antioxidant potential[4-6], tannins also have anticancer[7-9], antidiarrheal[6], antibacterial, antifungal, antiviral[4,5,10], anti-inflammatoire, cardioprotective and healing properties[5].

The bibliography suggests that tannins of *Combretum paniculatum* (Combretaceae), *Mallotus oppositifolius* (Euphorbiaceae) and *Ximenia americana* (Olacaceae), three medicinal plants from Côte d'Ivoire traditionally used in the treatment of many diseases[11] have not yet been subject to targeted investigations. The aim of this work was therefore to determine the content and antioxidant activity of tannins of these plants.

MATERIALS AND METHOD

Plant material

Plant material consists mainly of leaves of *Combretum paniculatum* (CP), *Mallotus oppositifolius* (MO) and *Ximenia americana* (XA). They were collected respectively at Anyama (5°29'40" North, 4°03'06" West), Bongouanou (6°38'55" North, 4°11'57" West) and Bouaké (7°41'00" North, 5°01'59" West) in June 2016, then identified at Centre National de Floristique (CNF) located at Félix Houphouët-Boigny University (Abidjan / Cocody) by botanists. Leaves of each plant were cleaned, dried in an air conditioned room (18°C) for 14 days and then pulverized using an electric grinder to provide powders for analysis.

Methodology

Tannins extraction

50 g of vegetable powder was macerated in 150 ml of acetone (80%) for 24 hours at room temperature, under permanent magnetic agitation. This operation was repeated 3 times with the

same marcs. After filtration on Büchner, the macerated products were collected and concentrated at 40°C in the rotary evaporator. Aqueous extract was kept in the refrigerator to remove lipophilic compounds. After decanting, CP^{ha}, MO^{ha}, and XA^{ha} crude extracts were obtained from *C. paniculatum*, *M. oppositifolius*, and *X. americana* leaves respectively. The crude extracts were taken in 3 × 60 ml of hexane and chloroform, respectively, to remove undesirable substances. After separation, aqueous fractions were treated with 3 × 60 ml ethyl acetate. The ethyl acetate fractions were concentrated to dryness in rotary evaporator to provide the dry extracts (CP^{ae}, MO^{ae} and XA^{ae}). The residual aqueous fractions were concentrated under reduced pressure, then dried in an oven at 50°C for 2 days to give dry aqueous extracts (CP^{aq}, MO^{aq} and XA^{aq})[12]. All six tannic selective extracts obtained were used for phytochemical and antioxidant analysis with respect to the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl).

Tannin detection by TLC

Tannins were revealed by TLC using iron perchloride ($FeCl_3$, 2% (w / v) and under UV / 365 nm light [13,14].

Tannins dosage

Total tannins were determined by permanganométrie[15] and spectrophotometry[16] respectively, modified by Sérémé *et al.* [4]. Condensed tannins were quantified by the method described by Heimler *et al.* [18].

Dosage of total tannins by permanganometry

This method is based initially on determination of proportions of all phenolic compounds after their oxidation. 1 g of a sample was taken in 100 ml of boiling water. This solution was heated in a water bath for 30 min with continuous stirring. After cooling and filtration, the volume was readjusted to 100 ml with distilled water. In a 1 l flask containing 10 ml of the decoction, 750 ml of distilled water and 25 ml of indigo sulfuric acid were added. The solution was titrated with potassium permanganate ($KMnO_4$ at 0.1N) under constant stirring until the reaction medium turned golden yellow. Under similar conditions, a blank (without extract) was titrated. Total phenolic compounds **X1** (in %) in dry matter were calculated according to formula **1** :

$$\mathbf{X1} = \frac{(\mathbf{V}-\mathbf{V1})\times 0.004157\times 250\times 100\times 100}{\mathbf{M}\times 25\times(100-\mathbf{W})} \qquad \textbf{(1)}$$

V (ml): volume of $KMnO_4$ (0.02 mol /l) consumed for the extract titration; **V1 (ml)**: volume of KMnO4 (0.02 mol / l) consumed for the blank titration; **M (g)**: organs mass; **0.004157 (g)**: tannins quantity corresponding to 1 ml of $KMnO_4$ (0.02 mol / l); **250 (ml)**: total volume of the extract; **100 (ml)**: total volume of extraction; **25 (ml)**: volume of extract taken for titration; **W (%)**: loss on drying of organs.

In a second step, the phenolic compound content without tannins (**X2**) was determined. To 20 ml of initial decocted water, 5 ml of gelatin (1%) in a sodium chloride solution (10% NaCl) was added, and the whole was kept at rest for 24 h in order to precipitate the tannins, which were recovered by filtration. 10 ml of filtrate were removed and titrated as before. The content of oxidized phenolic compounds excluding tannins (**X2**) was calculated (in %) according to equation **(1)** in which **V1** was replaced by **V2** (volume of KMnO4 used for titration after separation of tannins). Total tannin content (**X**) expressed as a percentage (%) was calculated by formula **(2)**:

$$\mathbf{X = X1 - X2} \qquad (2)$$

X1: content of total phenolic compounds oxidized by $KMnO_4$; **X2**: content of phenolic compounds oxidized by $KMnO_4$ without tannins.

Dosage of total tannins by spectrophotometry

After successive delipidation in Soxhlet with petroleum ether (3 h) and chloroform (3 h), 1 g of powder from each sample was macerated for 12 h in acetone (80%, 3 × 30 ml) with constant stirring. After filtration, 1 ml of macerate was taken from two test tubes (A and B). In tube (A), 5 ml distilled water, 1 ml ferric ammonium citrate (food additive E381) and 1 ml ammonia (NH_4OH) were added. In tube (B), 6 ml distilled water and 1 ml NH_4OH were added. After vortex agitation, the solution absorbance's was measured at 525 nm against the reference water. The quantification was carried out in relation to a calibration curve based on different concentrations of tannic acid [17]. Total tannin content (**T**), expressed as % mass tannic acid on a dry matter basis, was determined according to the relationship **(3)**

$$\mathbf{T = \frac{2C}{m} \times \frac{100}{100-H}} \qquad (3)$$

C (mg/ml): concentration of tannic acid in test solution, read on calibration curve; **m (g)**: mass of test sample; **H (%)**: water content of test sample.

Dosage of condensed tannins by spectrophotometry

3 g of leaves powder of each plant were decocted in 100 ml of distilled water for 30 min. After filtration, at 400 µl of decoction, 3 ml of methanolic solution of vanillin (4%) and 1.5 ml of concentrated hydrochloric acid (HCl) were added. After 15 min incubation, the solution absorbance was read at 500 nm. Concentrations of condensed tannins were deduced from a calibration curve established by a range of catechin concentrations from 31.25 to 250 µg/ml. The condensed tannin content was expressed in microgram catechin equivalent per milligram dry matter (µg ECAT/mg).

Antioxidant power measurement by spectrophotometry

Blois method[19], taken up by Kabran *et al.* [20] was used to measure antioxidant power of selective tannic extracts (CP^{ae}, MO^{ae}, XA^{ae} CP^{aq}, MO^{aq} and XA^{aq}). DPPH was solubilized in absolute ethanol to obtain a solution with a concentration of 0.3 mg/ml. Different concentration ranges (0.025; 0.05; 0.1; 0.25; 0.50 and 1 mg/ml) of each extract were prepared in same solvent. In dry, sterile tubes, 0.5 ml of extract and 1.5 ml of ethanolic solution from DPPH were introduced. After shaking, the tubes were kept away from light for 30 min. Mixture absorbance was measured at 517 nm against a blank of 0.5 ml absolute ethanol and 1.5 ml DPPH solution. Ascorbic acid (vitamin C) and gallic acid were used as reference antioxidants. The percentage reductions (%R) of DPPH were calculated according to formula **(4)**:

$$\%\mathbf{R} = \frac{(\mathbf{Ab}-\mathbf{Ae})}{\mathbf{Ab}} \times \mathbf{100} \qquad \mathbf{(4)}$$

Ab: blank absorbance, **Ae**: sample absorbance

For estimation of the antioxidant efficacy of selective tannin extracts, the median reduction concentrations of DPPH (CR_{50}) were determined using OriginPro 9.1 software.

Statistical analysis

All measurements were performed in triplicate and the tannin dosage results were expressed as mean ± standard deviation (Mean ± SD). One-factor analysis of variance (ANOVA ONE WAY) was used using Origin Pro 9.1 software. The difference between means was considered significant at 5%. If it was significant ($p < 0.05$), data were analyzed using the Tukey test (multiple comparison test).

RESULTS AND DISCUSSION

Our study focused on six selective tannic extracts

Tannic profile of selective extracts

The frontal ratios (Rf) and colorations of molecular spots obtained after interpretation of chromatograms, which indicate the presence of phytocompound families, are presented in table I. Condensed tannins and phenolic acids were highlighted under greenish-brown molecular fingerprints. Hydrolysable tannins were detected under blue-blackish colorations by $FeCl_3$ at 2% (w/v)[11,21,22]. The presence of flavonols and flavones was revealed under UV/365 nm as molecular spots blue, fluorescent yellow-green or orange-yellow molecular spots[23,24]. All ethyl acetate extracts contain hydrolysable and condensed tannins, phenolic acids and flavonoids type flavonol and flavone (Table I).

Table I: **Phytocompounds identified in different extracts with ethyl acetate**

Extracts	Rf (Color), Phytocompound identified
CP^{ae}	*0.05 (Br-Ve^d),* ***TC/Ap*** *; 0.21(J^a, G^b, Br-Ve^d),* ***TC/Ap*** *; 0.24(G^b, B^c, B-N^d),* ***TH*** *; 0.30 (J^a, G^b, B-N^d),* ***TH*** *; 0.32 (G^b, Ve^c, B-N^d),* ***TH*** *; 0.40 (J^a, G^b, Ve^c, Br-Ve^d),* ***TC/Ap*** *; 0.43 (B^c),* ***Fl*** *; 0.53 (J^a, G^b, R^c, Br-Ve^d),* ***TC/Ap*** *; 0.63 (J^a, G^b, R^c, Br-Ve^d),* ***TC/Ap*** *; 0.65 (J^a, G^b, R^c, Br-Ve^d),* ***TC/Ap*** *; 0.75 (G-J^a, B^c),* ***Fl***
MO^{ae}	*0.03(Jo^a, G^b, B-N^d),* ***TH*** *; 0.05(J^a, G^b, B^c, B-N^d),* ***TH*** *; 0.12(J^a, G^b, B-N^d),* ***TH*** *; 0.15(J^a, G^b, B^c, B-N^d),* ***TH*** *; 0.21(J^a, G^b, B^c, B-H^d),* ***TH*** *; 0.24(J^a, B^c),* ***Fl*** *; 0.28(G^b, Jo^c, Br-Ve^d),* ***TC/Ap*** *; 0.31(J^a, G^b, Ve^c, Br-Ve^d),* ***TC/Ap*** *; 0.41(J^a, G^b, Jo^c, Br-Ve^d),* ***TC/Ap*** *; 0.52(G^b, Jo^c, Br-Ve^d),* ***TC/Ap****; 0.61(G^b, B^c, Br-Ve^d),* ***TC/AP*** *; 0.65(J-Ve^c),* ***Fl****; 0.69(J-Ve^c),* ***Fl***
XA^{ae}	*0.04(Jo^a, G^b, B-N^d),* ***TH*** *; 0.06(J^a, G^b, J^c, B-N^d),* ***TH*** *; 0.1(J^a, G^b, B-N^d),* ***TH*** *; 0.14(J^a, G^b, Br^c, B-N^d),* ***TH*** *; 0.19(G^b, B-N^d),* ***TH*** *; 0.23 (J^a, G^b, J^c, B-N^d),* ***TH*** *; 0.28(J^a, G^b, B-N^d),* ***TH*** *; 0.35(Br^c),* ***Fl*** *; 0.39(J^a, G^b, Br-Ve^d),* ***TC/Ap*** *; 0.45(J^a, B^c),* ***Fl*** *; 0.50(G^b, Jo^c, Br-Ve^d),* ***TC/Ap*** *; 0.59(J^a, G^b, Jo^c, Br-Ve^d),* ***TC/Ap*** *; 0.70(J^a, G^b, B^c, Br-Ve^d),* ***TC/Ap*** *; 0.73(B^c),* ***Fl*** *; 0.78(J^a, G^b, B^c, M^d),* ***Fl*** *; 0.84(J-Ve^c),* ***Fl*** *; 0.88(B-F^c),* ***Fl***

J: *Yellow;* ***G***: *gray;* ***B***: *blue;* ***Jo***: *orange yellow;* ***Ve***: *green;* ***J-Ve***: *yellow green;* ***B-F***: *fluorescent blue;* ***B-N***: *blackish blue;* ***Br***: *brown;* ***Br-Ve***: *greenish brown;* ***M***: *brown;* ***R***: *red;* ***G-J***: *gray-yellow;* ***a***: *Compounds observed in visible;* ***b***: *Compounds revealed under UV at 254 nm;* ***c***: *Compounds revealed under UV at 365 nm;* ***d***: *Compounds revealed with 2% $FeCl_3$ solution;* ***TH***: *Hydrolysable tannin;* ***TC***: *Condensed tannin;* ***Fl***: *Flavonoid;* ***Ap***: *Phenolic acid*

The aqueous fractions (CP^{aq}, MO^{aq}, XA^{aq}) contain condensed tannins and phenolic acids, except for *M. oppositifolius* leaves. Hydrolysable tannins are present in all selective extracts. Flavonols and flavones were visualized only in *C. paniculatum* and *M. oppositifolius* (Table II).

Table II: **Phytocompounds identified in the various aqueous extracts**

Extracts	Rf (Color), Phytocompound identified
CP^{aq}	*0.03(J^a, G^b, B^c, B-N^d),* ***TH****; 0.19(J^a, G^b, B^c, B-N^d),* ***TH****; 0.29(J^a, G^b, B^c, B-N^d),* ***TH****; 0.42(J^a, G^b, J^c, Br-Ve^d),* ***TC/Ap*** *; 0.47(J^a, G^b, J^c, Br-Ve^d),* ***TC/Ap*** *; 0.56(B^c),* ***Fl*** *; 0.65(J^a, G^b, B^c, J^d),* ***Fl***
MO^{aq}	*0.04(J^a, G^d),* ***TH*** *; 0.14(J^a, G^b, B-N^d),* ***TH****; 0.32 (G^b, J^c, B-N^d),* ***TH****; 0.47(G^b, B^c, J^d),* ***Fl****; 0.62(B^c, J^d),* ***Fl***
XA^{aq}	*0.15(J^a, G^b, B^c, B-N^d),* ***TH****; 0.30 (J^a, G^b, J^c, B-N^d),* ***TH****; 0.37(J^a, G^b, J^c, B-N^d),* ***TH****; 0.47(G^b, Ve^c, Br-Ve^d),* ***TC/Ap*** *; 0.58(G^b, B^c, Br-Ve^d),* ***TC/Ap****; 0.66(G^b, Br-Ve^d),* ***TC/Ap*** *; 0.74(G^b, Br-Ve^d),* ***TC/Ap***

J: *Yellow;* ***G***: *gray;* ***B***: *blue;* ***Ve***: *green;* ***B-N***: *blackish blue;* ***Br-Ve***: *greenish brown;* ***a***: *Compounds observed in visible;* ***b***: *Compounds revealed under UV at 254 nm;* ***c***: *Compounds revealed under UV at 365 nm;* ***d***: *Compounds revealed with 2% $FeCl_3$ solution;* ***TH***: *Hydrolysable tannin;* ***TC***: *Condensed tannin;* ***Fl***: *Flavonoid;* ***Ap***: *Phenolic acid.*

In conclusion, the phytochemical screening aimed to establish a preliminary tannic profile ofr six selective extracts (CP^{ae}, MO^{ae}, XA^{ae} CP^{aq}, MO^{aq} and XA^{aq}). It appears that tannins extraction was conducted selectively. Indeed, the results obtained and recorded in tables I and II show the presence of other phenolic phytoconstituents, notably phenolic acids and flavonoids. Moreover, it has been shown that *C. paniculatum*, *M. oppositifolius* and *X. americana* leaves contain hydrolysable and condensed tannins, and that these are selectively extractable.

Totals tannins content of selective extracts

Permanganometry and spectrophotometry were used to quantify the tannins. Quantitative method by permanganometry gave significant total tannin contents (41.03±0.77, 20.03±0.4 and 18.76±0.89%), respectively in MO, XA and CP (Figure 1).However, *M. oppositifolius* leaves were found to be the richest in tannins, which seems to be explained by an abundance of phytophenols oxidizable by $KMnO_4$[15]. Indeed, the proportion of total polyphenols (cf. equation 2) recorded in *M. oppositifolius* (48.59±0.44%) is higher than that of *X. americana* (37.13±0.77%) and *C. paniculatum* (27.31±0.89%).

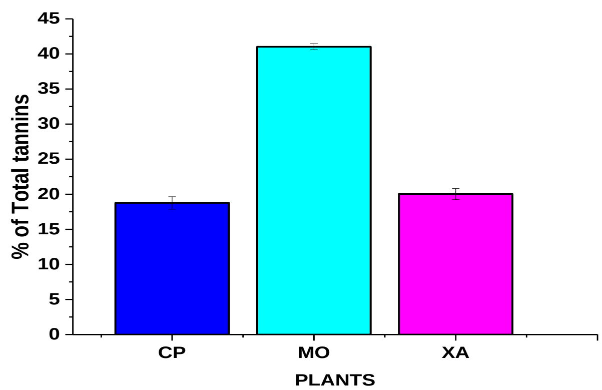

Figure 1: Total tannins content determined by permanganometry

Total tannin quantification by spectrophotometry also showed that *M. oppositifolius* leaves are richer in tannins with an estimated value of 12.3648±0.0004%, followed by *X. americana* (11.7974±0.0001%) and *C. paniculatum* (11.6026±0.0008%) (Figure 2).

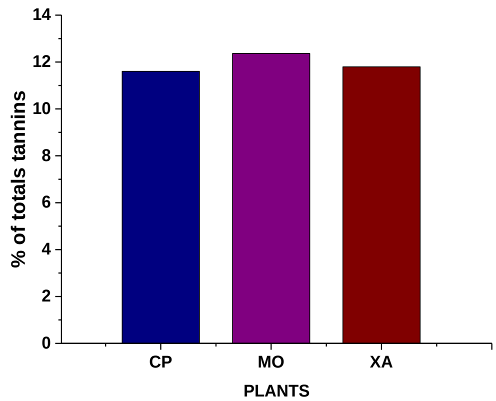

Figure 2: Total tannins content determined by spectrophotometry

However, a significant difference between total tannin contents clearly emerges from the results obtained with the two quantification methods used (Figures 1 and 2). This difference could be explained, on one hand, by selectivity of each method with respect to the tannins present in a mixture with similar substances, and on the other hand, by tannins oxidation by $KMnO_4$ (titrimetric analysis by permanganometry) compared to the fixation of ferric ions (Fe^{3+}) on the tannin molecules (analysis by spectrophotometry). Indeed, the existence of steric encumbrance around the fixation sites, seems to cause less complex formation, which would lead to a low percentage of total tannins. Similar observations were made by other researchers when they dosed the tannins in *Acacia nilotica* leaves by two different methods. Reed *et al.* [25] obtained 49.1% tannin content by gravimetric ytterbium precipitation analysis, while Sérémé *et al.* [17] recorded 8.5% tannin content in the same plant by spectrophotometric analysis. Overall, tannin estimation in a plant matrix depends on the type of quantification analysis used.

Condensed tannins content

X. americana leaves showed significantly higher contents of condensed tannins (234.135 ± 0.003 µgECT / mg) compared to those of *C. paniculatum* (37.731 ± 0.001 µgECT / mg) and *M. oppositifolius* (19.082 ± 0.001 µgECT / mg) (Figure 3).

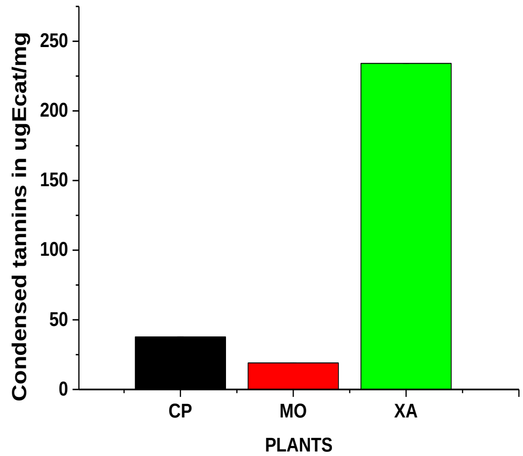

Figure 3: Condensed tannins content

By comparing our results with those reported in the literature [26,27], we suggest that *X. americana* could be classified as a plant with condensed tannins.

Antioxidant activity of selective tannic extracts

Antioxidant activity of the six selective tannic extracts (CP^{ae}, MO^{ae}, XA^{ae}, CP^{aq}, MO^{aq}, and XA^{aq}) was estimated by spectrophotometry against DPPH. Figure 4 shows the reduction power (%R) of DPPH by all the selective tannic extracts at variable concentrations, compared to two reference antioxidants (vitamin C (VIT C) and gallic acid (GAL ACID)). This suggests that the reduction power of DPPH is dependent on concentration. However, at the lowest concentration (0.025 mg/ml), just like gallic acid (66.37%), only aqueous selective tannic extract from *X. americana* leaves (XA^{aq}) shows a %R of DPPH (47.46%). In addition, we note that at 0.25, 0.5 and 1 mg/ml all extracts recorded %R from DPPH (92.86±0.003 - 96.39±0.002%) close to that of vitamin C (97.86±0.004%) and gallic acid (95.91±0.0008%). This highly significant scavenging activity of DPPH seems to be due to the synergistic action of tannins contained in the selective extracts. Indeed, quantitative screening has shown that these extracts are rich in tannins (Figures 1-3).

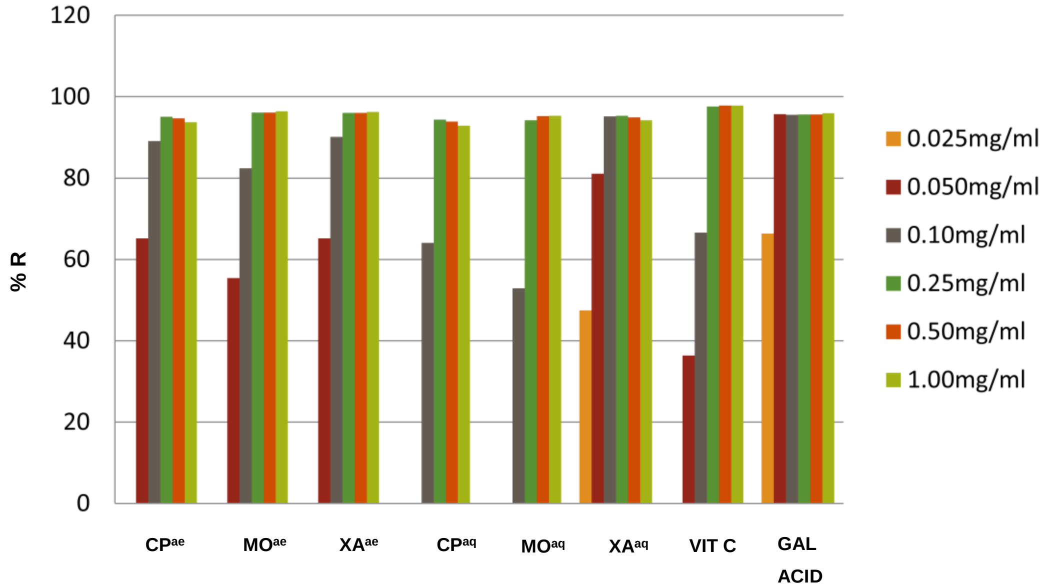

Figure 4: Percentage reduction of DPPH by selective tannic extracts

In order to evaluate critically the antioxidant efficacy of these extracts, CR_{50} of each extract was determined. This parameter represents the concentration at which 50% of maximal antioxidant effect of extract is observed. The lower this median concentration is, the more active the extract has a good antioxidant activity [28]. CR_{50}s determined (Table III) show that the selective tannic extracts XA^{aq}, MO^{ae}, XA^{ae}, and CP^{ae} have a more significant antioxidant efficacy of DPPH than vitamin C. However, among these extracts, the best antioxidant efficacy is that from XA^{aq}. Among phytophenols, tannins are known as excellent natural antioxidants[3,29-31]. Thus, it seems evident that, overall, the antioxidant activity exhibited by *C. paniculatum, M. oppositifolius*, and *X. americana* would explain their recurrent use in the traditional care of tumor patients[7,9,11].

Table III: **CR_{50} (mg / ml) of selective tannic extracts**

Combretum paniculatum		***Mallotus oppositifolius***		***Ximenia americana***		**Vit C**	**Gal acid**
Ext	CR_{50}	Ext	CR_{50}	Ext	CR_{50}	CR_{50}	CR_{50}
CP^{ae}	0.046±0.004	MO^{ae}	0.044±0.005	XA^{ae}	0.045±0.005	0.077±0.005	<0.025±0.004
CP^{aq}	0.089±0.008	MO^{aq}	0.096±0.005	XA^{aq}	0.031±0.003	Idem	Idem

Ext: Extracts; **Vit C**: Vitamin C; **Gal acid**: Gallic acid

CONCLUSION

Determination of tannin content by permanganometry and spectrophotometry in the leaves of three medicinal plants from the floristic biodiversity of Côte d'Ivoire, namely *Combretum paniculatum*, *Mallotus oppositifolius* and *Ximenia americana*, allowed to identify them as tanniferous species. The estimation of CR_{50} from the reduction percentages of DPPH, showed an antioxidant efficacy of selective tannic extracts from all plants, which seems to be governed by their tannin richness. Antioxidant efficacy of these plants could be considered as a rational justification for their recurrent use in traditional medicinal practice in the treatment of several pathologies including cancers. The tannin fractions derived from these plants would therefore deserve special attention with a view to designing improved phyto-preparations for the primary health care coverage of populations.

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