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Antioxidant activity and total polyphenol and flavonoid contents of *Cissus doeringii* Gilg & M. Brandt (Vitaceae) and *Diospyros mespiliformis* Hochst. ex DC. (Ebenaceae), used for treating malaria

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Abstract

Cissus doeringii (Vitaceae) and *Diospyros mespiliformis* (Ebenaceae) are two plants traditionally used to treat malaria that generates strong oxidative stress. This study was conducted to evaluate the *in vitro* antioxidant activity and the total polyphenol and flavonoid contents of the aqueous and methanolic extracts of leaves of these two plants. The antioxidant activity of extracts was evaluated by the free radical scavenging methods (DPPH and ABTS). Polyphenol and flavonoid contents of the extracts were determined by the spectrocolorimetric method. The results of the antioxidant activity revealed that the DPPH radical inhibition percentages of the extracts ranged from 34.95 ± 3.4 to 70.84% with IC_{50} ranging from 5.52 to 36.59 µg/mL and those of the ABTS radical from 52.98 ± 6,69 to 81.32 ± 3.08% with IC_{50} between 5.98 and 34.12 µg/mL. The methanolic extract of the leaves of *Cissus doeringii* showed the best antiradical activity (ABTS) with an IC_{50} of 5.98 µg/mL, in comparison to those of the references, ascorbic acid (5.30 µg/ml) and trolox (5.73 µg/ml). The antioxidant capacities of the extracts ranged from 59.79 ± 7.55 to 68.26 ± 5.76 mg of TEg^{-1} dry matter. The polyphenol contents were between 336.36 ± 0.58 and 466.67 ± 0.58 mg EAG/g of extract and the total flavonoid contents between 202.14± 0.26 and 254.06 ± 0.26 mg ECA/g extract. These two plants could be sources of natural antioxidants able to prevent the oxidative stress associated with *Plasmodium* infection and other diseases.

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Introduction

Excessive production of free radicals can become toxic to the major components of the cell (lipids, proteins and nucleic acids) and consequently gives rise to oxidative stress (Morales-González, 2013). This oxidative stress, generally due to reactive oxygen species (ROS), appears to be causes of a broad spectrum of chronic and degenerative diseases such as heart disease, cancer, diabetes and accelerates the aging process (Dichi *et al.*, 2013). They are even implicated in pathologies such as gastric ulcers, bacterial infections and malaria (Gülçin *et al.*, 2006). In the case of malaria infection, high oxidative stress is generated during the acute phase. This is thought to be due to the metabolism of iron-rich components, an increase in the production of ROS (Hans *et al.*, 2009) and a decrease in antioxidant enzymes and certain compounds such as catalase, GSH, GSH peroxidase and SOD (Müller, 2015). This oxidative stress is generally controlled by treatment with antioxidants. Synthetic antioxidants such as Butyl Hydroxy Anisole (BHA) and Butyl Hydroxyl Toluene (BHT) exist and are certainly very effective.

However, they are susceptible to manifest side effects and also toxic (Manian *et al.*, 2008 ; Liu and Yang, 2018). It is therefore interesting to look for natural antioxidants that are effective from plants and have almost no side effects. Many studies have shown that plants possess antioxidant properties largely due to phenolic compounds and flavonoids in particular (Djeridane *et al.*, 2006 ; Leja *et al.*, 2013). Thus, the determination of the antioxidant activity of plant extracts is most often associated with that of the content of total phenolic compounds and total flavonoids in order to establish a correlation between phytocompounds and antioxidant activity.

This study was conducted in order to explore the *in vitro* free radical scavenging potential and total phenolic compound and total flavonoids content of *Cissus doeringii* Gilg & M.Brandt (Vitaceae) and *Diospyros mespiliformis* Hochst ex A.DC (Ebenaceae), two medicinal plants used in Côte d'Ivoire to treat malaria.

Material and methods

The plant material consisting of leaves of *Cissus doeringii* Gilg & M.Brandt and *Diospyros mespiliformis* Hochst ex A.DC was collected in the locality of Bouaké in the centre of Côte d'Ivoire in April 2019 after an ethnobotanical survey among traditherapists and herbalists in the Abidjan District (Sylla *et al.*, 2018).

Preparation of crude extracts

The leaves were air-dried (21°C) for two weeks and ground to obtain fine powders. Three extraction methods were used in this study : decoction and aqueous maceration based on traditional preparations followed by extraction with increasing polarity organic solvents for optimizing the extraction and concentrating the compounds.

Decoction was made by dissolving 25 g of plant powder in 250 mL of distilled water and heating the mixture at 100°C for 15 min.

Aqueous macerate was obtained by dissolving 25 g of plant powder in 250 mL of distilled water in an Erlenmeyer flask under mechanical stirring (175 rpm) for 24 hours. All extracts were filtered, concentrated using a rotary evaporator at a temperature of 40°C and dried in an oven at 45°C.

For successive extraction with solvents of increasing polarity, 25 g of each powder was macerated in 250 mL of dichloromethane under mechanical stirring for 24 hours.

After filtration, the residual marc was dried at room temperature, weighed, then taken up in methanol (250 mL) under mechanical stirring for 24 h. The mixture was filtered, concentrated in a rotary evaporator at 40°C and dried in an oven at 45°C.

The dry extracts obtained were then weighed and the yield was calculated using the following formula :

$$R(\%) = \frac{m}{M} * 100$$

R = Extraction yield in % ; m = Mass of the extract ;

M = Mass of the plant powder.

Evaluation of the anti-radical activity of the extracts

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging test

The anti-free radical activity of plant extracts was measured using DPPH assay according to the method described by Sanchez-Moreno *et al.* (2002). A volume of 2.5 mL of 0.04% methanolic DPPH solution was added to 100 µL of the different dilutions (1000-3.75 µg/mL) of each extract and the standards. After homogenization, the mixtures were incubated at room temperature (25°C) in the dark for 30 minutes. Absorbance was measured at 517 nm, against a "blank". Tests were performed in triplicate and absorbances were averaged. Ascorbic acid and trolox were used as standards under the same conditions as the samples. A calibration curve was plotted with trolox concentrations ranging from 800 to 50 µg.mL^{-1}. The percentage of DPPH radical recovered or percentage of inhibition was calculated with the following formula :

$$\text{DPPH Inhibition (\%)} = [(A_0 - A_1) / A_0] \times 100$$

With A_0 = absorbance of the blank and A_1 = absorbance of the extract after 30 min of incubation.

The IC_{50} (50% Inhibitory Concentration) corresponding to the concentration of the sample tested required to reduce the DPPH radical by 50%, were determined graphically for each sample (Diomande *et al.*, 2018) by linear regressions of the graphs plotted from the percentages of inhibition as a function of different concentrations tested.

The antioxidant capacity of the extracts, expressed in mg of Trolox Equivalent/g of dry matter (mgTE.g^{-1} DM) was determined using the formula proposed by Wangcharoen and Morasuk (2007):

$$\text{Value of DPPH (mg de TE.g}^{-1}\text{DM)} = \frac{\left[\frac{A_1 - A_0}{\text{slope}}\right]\left[\left(\frac{V}{v}\right)\right]}{[m][100]} \times r$$

With A_0 = absorbance of blank; A_1 = absorbance of the extract; slope = slope of the equation line expressing the absorbance values as a function of trolox concentrations ; v = volume of extract taken for the test ; V = total volume of extract prepared to obtain the stock solution ; m = mass of sample taken to prepare the stock solution ; 1000 = conversion factor ; r = yield ; DM = dry matter ; TE = Trolox equivalent.

ABTS Radical Trapping Test (2,2'-azynobis-[3-ethylbenzothiazoline-6-sulfonic acid])

The anti-free radical activity of the plant extracts was measured using the [2.2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS) assay according to the method described by Choong *et al.* (2007). The $ABTS^+$ radical-cation was produced by the reaction of 7 mM ABTS (4.06 mg of ABTS powder in 1 mL of distilled water) and 2.42 mM of potassium persulfate (0.7 mg of potassium persulfate in 1 mL of distilled water) in a 1:1 (v/v) ratio. The mixture was then incubated in the dark at room temperature for 12 to 16 hours. On the day of testing, this ABTS+ solution was diluted with methanol to give a solution with an absorbance of 1.2±0.4 at 734 nm. A volume of 3 mL of the diluted $ABTS^+$ solution was added to 100 µL of the different dilutions (1000-3.75 µg/mL) of each extract and of the standards to be tested. After shaking, the mixture was incubated in the dark at room temperature (25°C) for 30 min. The residual absorbance of the $ABTS^+$ radical was then measured at 734 nm with a UV-visible spectrophotometer against a "blank". The tests were carried out in triplicate and absorbances were averaged. Ascorbic acid and trolox were used as standards under the same conditions as the samples. A calibration curve was plotted with trolox concentrations ranging from 800 to 50 µg.mL^{-1}.

The percentage of ABTS radical recovered or percentage of inhibition was calculated with the following formula:

$$\text{ABTS Inhibition (\%)} = [(A_0 - A_1) / A_0] \times 100$$

With A_0 = absorbance of the blank and A_1 = absorbance of the extract after 30 min of incubation.

The ICs_{50} were determined graphically as before. The antioxidant capacity of the extracts, expressed in mg of Trolox Equivalent/g of dry matter (mgTE.g^{-1}DM) was determined using the formula proposed by Wangcharoen and Morasuk (2007):

$$\text{Value of ABTS (mg de TE.g}^{-1}\text{DM)} = \frac{\left[\frac{A_1 - A_0}{\text{slope}}\right]\left[\left(\frac{V}{v}\right)\right]}{[m]\,[1000]} \times r$$

With: A_0 = absorbance of blank; A_1 = absorbance of the extract ; slope = slope of the equation line expressing the absorbance values as a function of trolox concentrations ; v = volume of extract taken for the test ; V = total volume of extract prepared to obtain the stock solution ; m = mass of the sample taken to prepare the stock solution ; 1000 = conversion factor ; r = yield ; DM= dry matter ; TE = Trolox equivalent.

Determination of polyphenol and total flavonoid content

Total polyphenol content

The total phenol contents of the plant extracts were determined using the Folin-Ciocalteu method (Djeridane et al., 2006 ; Li et al., 2007 ; Bédié et al., 2011). To a volume of 500 µL of each plant extract (100 µg/mL) were respectively added 2500 µL of Folin-Ciacolteu diluted 1/10 in distilled water and 2000 µL of sodium carbonate (1M). The mixture was incubated at room temperature for 15 min in a water bath at 50°C. Then the tubes were rapidly cooled in ice-cold water, the optical densities (OD) were read against a blank in a spectrophotometer at 760 nm. A calibration curve was produced using gallic acid as a positive control at concentrations ranging from 200 to 0 µ/mL under the same conditions as the samples. The total phenol contents of the extracts are expressed as mg gallic acid equivalents per gram of extract (mg EAG/g of extracts).

Total flavonoid content

The flavonoid content of the extracts was determined using the aluminum trichloride colorimetric method (Chang et al., 2002). To a volume of 500 µL of each plant extract (100 µg/mL) were added 1500 µL of distilled water and 150 µL of 5% sodium nitrite (NaNO2). After 5 min, 150 µL of 10% (w/v) aluminum trichloride ($AlCl_3$) were added to the mixture. After 6 min incubation at room temperature, 500 µL of 4% sodium hydroxide (NaOH) were added. The mixture was stirred to homogenize the contents.

The absorbance of the solutions was determined at 510 nm against a blank. A calibration curve was produced using catechin as a positive control at concentrations ranging from 100 to 0 µg/mL under the same conditions as the samples. The total flavonoid content of the extracts was expressed as mg of catechin equivalents per gram of extract (mg ECA/g of extracts).

Statistical analyzes

For analyzing the results, Microsoft Excel 2007 software was used to enter the data which was then exported to Statistica 7.1 for statistical processing. The results were presented as mean ± standard deviation. The difference between the means was determined by one way analysis of variance (ANOVA). Significant differences between the means were determined by the Fisher's test at the theoretical threshold α = 5%. The correlation between the activities studied is given by regression analysis. The correlation coefficient $R^2 \geq 0.90$ was used as the highest correlation value.

Results

Yields of prepared extracts

The yield of the extractions varied according to the plant species, the nature and the polarity of the solvent used (Fig.1). Aqueous leaf extracts produced the highest yields of 6.96% and 6.07% for *C. doeringii* and *D. mespiliformis* respectively, compared to 4.76% and 4.18% for the methanol extractions.

Anti-free radical activity of the extracts

DPPH radical inhibitory powers

The percentage of DPPH inhibition, the IC_{50} values and antioxidant capacities of the extracts are given in Table 1. The free radical scavenging activity of the 4 extracts tested was dose-dependent, with a strong correlation ($R^2 \geq 0.90$) between the concentration and the percentage of DPPH radical inhibition for the extracts tested (Fig. 2). All the extracts tested showed varying degree of activity between 34.95 and 70.84%. The most active extracts were the methanolic extracts of *C. doeringii* and *D. mespiliformis* leaves with DPPH radical inhibition percentages of 72.84±3.51

and 68.61±5,92% respectively, statistically equal to those of the two reference products, trolox (78.73%) and ascorbic acid (81.32±3.08%) (P > 0.05).

Table 1. DPPH radical inhibition percentages, inhibitory concentrations 50% (IC_{50}) and Antioxidant capacity of studied plants and reference compounds.

Plant species	Extracts	Inhibition of the radical DPPH (%) ± SD	CI_{50} (µg/mL)	ACV DPPH (mgTEg^{-1} DM)± SD
Cissus doeringii	Methanolic	72.84±3.51^{a}	5.98	70.43±3.49^{a}
	Decocted	34.95±3.45^{b}	36.59	47.80±4.67^{b}
Diospyros mespiliformis	Methanolic	68.61±5.92^{a}	12.43	67.4±67.09^{a}
	Macerate	50.96±5.66^{b}	19.12	65.00±6.88^{a}
Vitamine C		78.73±4.25^{a}	4.31	nd
Trolox		76.84±2.21^{a}	4.86	nd
Statistical parameters	Dl	5		3
	F	13.11		7.21
	P	<0.001		<0.001

TE = Trolox equivalent; IC_{50}: Concentration of extract inhibiting 50% of the DPPH radical; mgTEg-1 MS: mg Trolox. Equivalent ; ACV = Antioxidant Capacity Value ; a = high activity ; b = average activity ; DPPH : 2,2'-Diphenyl-1-picryl hydrazyl, SD = Standard Deviation ; Values with the same letters are not significantly different.

The IC_{50} values of the extracts tested ranged from 5.52 to 36.59 µg/mL (Table 1). The methanolic extract of *C. doeringii* leaves had the highest activity (5.52 µg/mL) very close to those of ascorbic acid (4.31 µg/mL) and trolox (4.86 µg/mL).The antioxidant capacities of the extracts obtained with the DPPH test ranged from 47.80 to 70.43±3.49 mgET.g^{-1} DM (Table 1).

The methanolic extracts of *C. doeringii* leaves (70.43±3.49 mgET.g^{-1} DM) and *D. mespiliformis* leaves (67.4±67.09 mgET.g^{-1} DM) had the highest antioxidant capacities (P > 0.05).

ABTS radical inhibitory powers

The ABTS radical inhibitory powers of plants, expressed as percentages of inhibition, are given in Table 2. The anti-free radical activity exerted by the six extracts tested on the ABTS radical was also dose-dependent (Fig. 3). All the extracts tested showed varying degrees of activity, with percentages of ABTS inhibition ranging from 52.98±6.69 and 81.32±3.08%. The methanolic extracts of *C. doeringii* and *D. mespiliformis* leaves were the most active, at 81.32±3.08% and 68.37±5.77% respectively, statistically equal to those of trolox (88.73±6.67%) and ascorbic acid (90.75±4.38%) (P > 0.05).

Table 2. Percentages of the ABTS radical inhibition, inhibitory concentrations 50% (IC_{50}) and antioxidant capacity of active plants and reference compounds.

Plant species	Extracts	Inhibition of the radical ABTS (%) ±SD	CI_{50} (µg/mL)	ACV ABTS (mgTEg^{-1} DM)±SD
Cissus doeringii	Methanolic	81.32±3.08^{a}	5.98	68.26±5.76^{a}
	Decocted	52.98±6.69^{b}	34.12	59.79±7.55^{b}
Diospyros mespiliformis	Methanolic	68.37±5.77^{ab}	13.19	67.34±2.55^{b}
	Macerate	64.97±6.43^{b}	18.54	65.84±6.52^{a}
Vitamine C		88.73±6.67^{a}	5.3	nd
Trolox		90.75±4.38^{a}	5.73	nd
Statistical parameters	Dl	5		3
	F	11.02		6.31
	P	<0.001		<0.001

TE = Trolox Equivalent; IC_{50}: Concentration of extract inhibiting 50% of the ABTS radical ; mgTEg^{-1} MS= mg Trolox Equivalent per gram of dry matter ; ACV = Antioxidant Capacity Value ; SD= Standard Deviation ; a = high activity ; b = medium activity ; F= Fisher statistic; P= probability ; Values with the same letters are not significantly different.

The IC_{50} values of the extracts tested ranged from 5.98 to 34.12 µg/mL (Table 2). The methanolic extract of *Cissus doeringii* leaves showed the best activity, with an IC_{50} of 5.98 µg/mL superposable with those of ascorbic acid (5.3 µg/mL) and trolox (5.73 µg/mL). The antioxidant capacities of the extracts obtained in the ABTS test ranged from 47.80±4.67 to 68.26±5.76 mg ETg^{-1} DM (Table 2). *C. doeringii* (68.26±5.76 mg $ET.g^{-1}$ DM) and *D. mespiliformis* (67.34±2.55 mg $ET.g^{-1}$ DM) had the highest antioxidant capacities ($P > 0.05$). These results confirm the high antioxidant capacity of these two plants, already demonstrated in the case of DPPH.

Table 3. Total polyphenol and flavonoid contents of active plant extracts.

Plant species	Extracts	Polyphenol content (mg EAG /g) ±SD)	Flavonoids Content (mg ECA/g) ± SD)
Cissus doeringii	Methanolic	466.67 ±0.58^{a}	254.06±0.26^{a}
	Decocted	403.03 ±0.58^{c}	230.87±0.45^{b}
Diospyros mespiliformis	Methanolic	442.42 ±0.58^{b}	227.71±1.20^{c}
	Macerate	336.36 ±0.58^{e}	202.14±0.26^{d}
Statistical parameters	Dl	3	3
	F	9522	999.8
	P	<0.001	<0.001

F = Fisher statistic ; P = probability ; SD = Standard Deviation ; GAE= Gallic Acid Equivalent ; CAE = Cathechin Equivalent ; Values with the same letters are not significantly different.

Total polyphenol and flavonoid content

Total polyphenol content

Total polyphenol contents of the active plant extracts are given in Table 3. The results showed that the methanolic extracts of *D. mespiliformis* (466.67 ±0.58 mg EAG/g of extract) and *C. doeringii* (442.42 ±0.58 mg EAG/g of extract) were the richest in phenols. The difference in polyphenol contents according to plant and the solvent used was highly significant ($P < 0.001$).

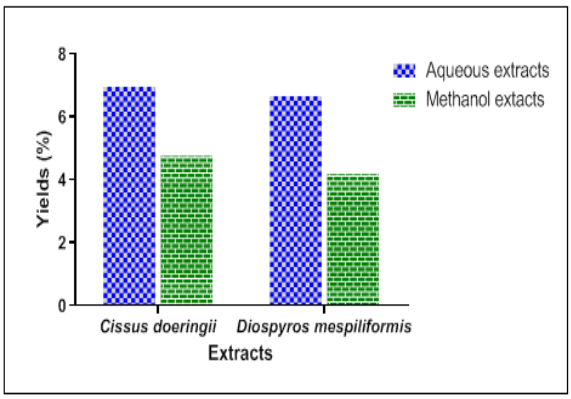

Fig. 1. Histogram of extraction yields for plant organs.

Total flavonoid content

The flavonoid contents of the various plant extracts are given in Table 3. These results showed that the methanolic and aqueous extracts of *C. doeringii* (254.06±0.26 mg ECA/g of extract and 230.87±0.45 mg ECA/g of extract) were the richest in flavonoids. The difference in flavonoid content according to plant and solvent used was highly significant ($P < 0.001$).

A strong linear correlation was established between the total polyphenol content and the antioxidant activity of different active extracts, with R^2/ABTS = 0.92 (Fig. 4A). However, the linear correlation established between the flavonoid content and the antioxidant activity of the different extracts studied was weak, with R^2 /ABTS = 0.22 (Fig. 4B).

Discussion

In this study, the antioxidant activity of methanolic and aqueous extracts of *C. doeringii* and *D. mespiliformis*, two plants used in the Ivorian pharmacopoeia to treat malaria, was evaluated. The methanolic extracts of the two plants tested showed good antioxidant activity with respectively, percentages of inhibition of the DPPH radical = 72.84±3.51%; 68.61±5.92% and the ABTS radical = 81.32±3.08%; 68.37±5,77%. The ICs_{50} of *C. doeringii* and *D. mespiliformis* were DPPH = 5.52 µg/mL; 12.43 µg/mL and ABTS = 5.98 µg/mL; 13.19 µg/mL respectively. This activity was comparable to those of

the two reference products, trolox and ascorbic acid, which respectively showed DPPH radical inhibition percentages of 76.84±2.21% and 78.73±4.25 ($p > 0.05$) with IC_{50} values of 4.86 µg/mL and 4.31 µg/mL. These results are comparable to those obtained by Ehouman *et al.* (2016) for the methanolic extract of *C. doeringii*, with a percentage inhibition of 81.13±1.42% and an IC_{50} of 5.70 µg/mL using ABTS assay. This plant is used as an analgaesic, restorative and against malaria (Eklu-Natey *et al.*, 2011). Its high antioxidant activity could be linked to its traditional uses, as it is known that free radicals are involved in the genesis of pain and fatigue (Muñoz *et al.*, 2011), rheumatic inflammation (Jomova and Valko, 2011) and are an aggravating factor in malaria (Gehrke *et al.*, 2013).

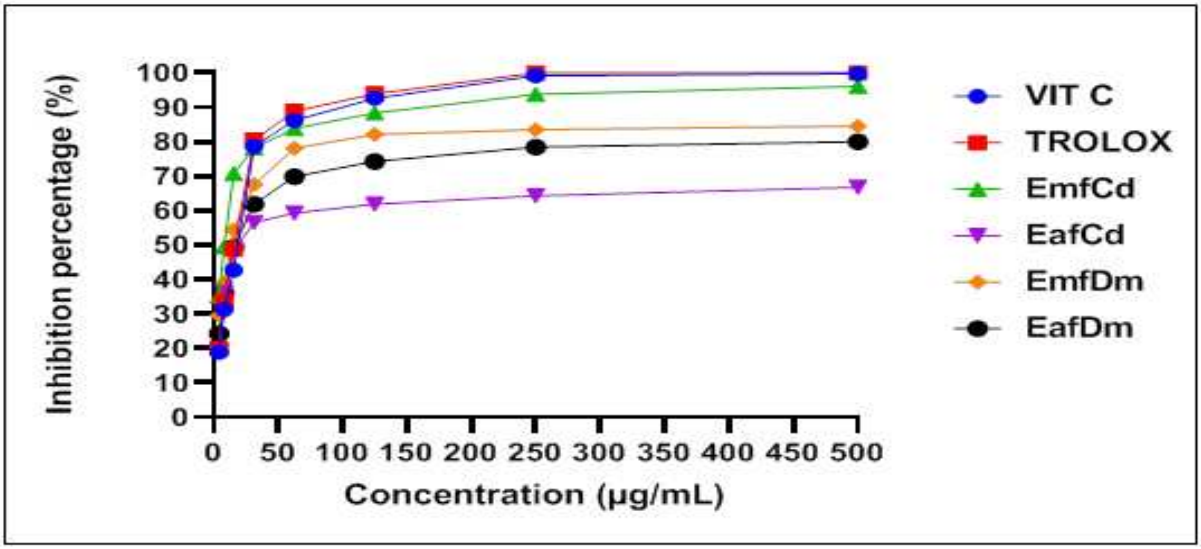

Fig. 2. Evolution of the reducing power of the DPPH radical in extracts of plants studied according to their concentration.

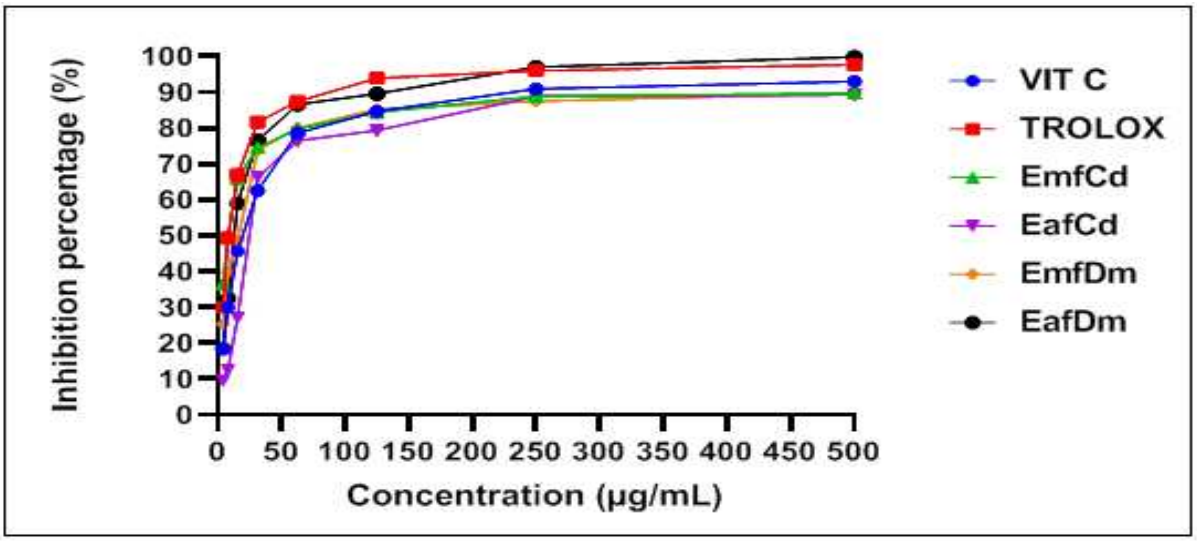

Fig. 3. Evolution of the reducing power of the ABTS radical of extracts of plants studied according to their concentration.

In the case of malaria, strong oxidative stress is generated in the host not only by the parasite through membrane lysis of red blood cells (Griffiths *et al.*, 2001) but also by the defense system, neutrophils, which activated by the parasite causes destruction of endothelial cells (Hemmer *et al.*, 2005). This phenomenon contributes to their destruction and anemia. The antioxidant activity of plants could help

to reduce oxidative stress linked to *Plasmodium* invasion and thus facilitate the success of antimalarial treatment. Methanolic and aqueous extracts of *D. mespiliformis* leaves showed good free radical scavenging activities, with ICs_{50} values of 13.19 µg/mL and 18.54 µg/mL respectively. This plant is traditionally used against fever, malaria and as a tonic (Eklu-Natey *et al.*, 2011). Its antioxidant properties studied in this work may justify their traditional use to treat all these diseases and improve patients 'health. Several molecules with antioxidant properties such as quercetin, hypericin, gallic acid, ascorbic-acid, tocopherols and tocotrienols have been isolated from plants (Cakir *et al.*, 2003; Kumaran and Karunakaran, 2007). It is therefore laudable to look for effective natural antioxidants, especially as synthetic antioxidants such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) are certainly very effective, but are likely to have side-effects and even toxicity (Manian *et al.*, 2008). The high levels of total polyphenols and flavonoids found in the leaves of *C. doeringii* and *D. mespiliformis* and the existence of a good linear correlation between total polyphenols and antioxidant activity of their extracts showed that total polyphenols may be responsible for this antioxidant activity.

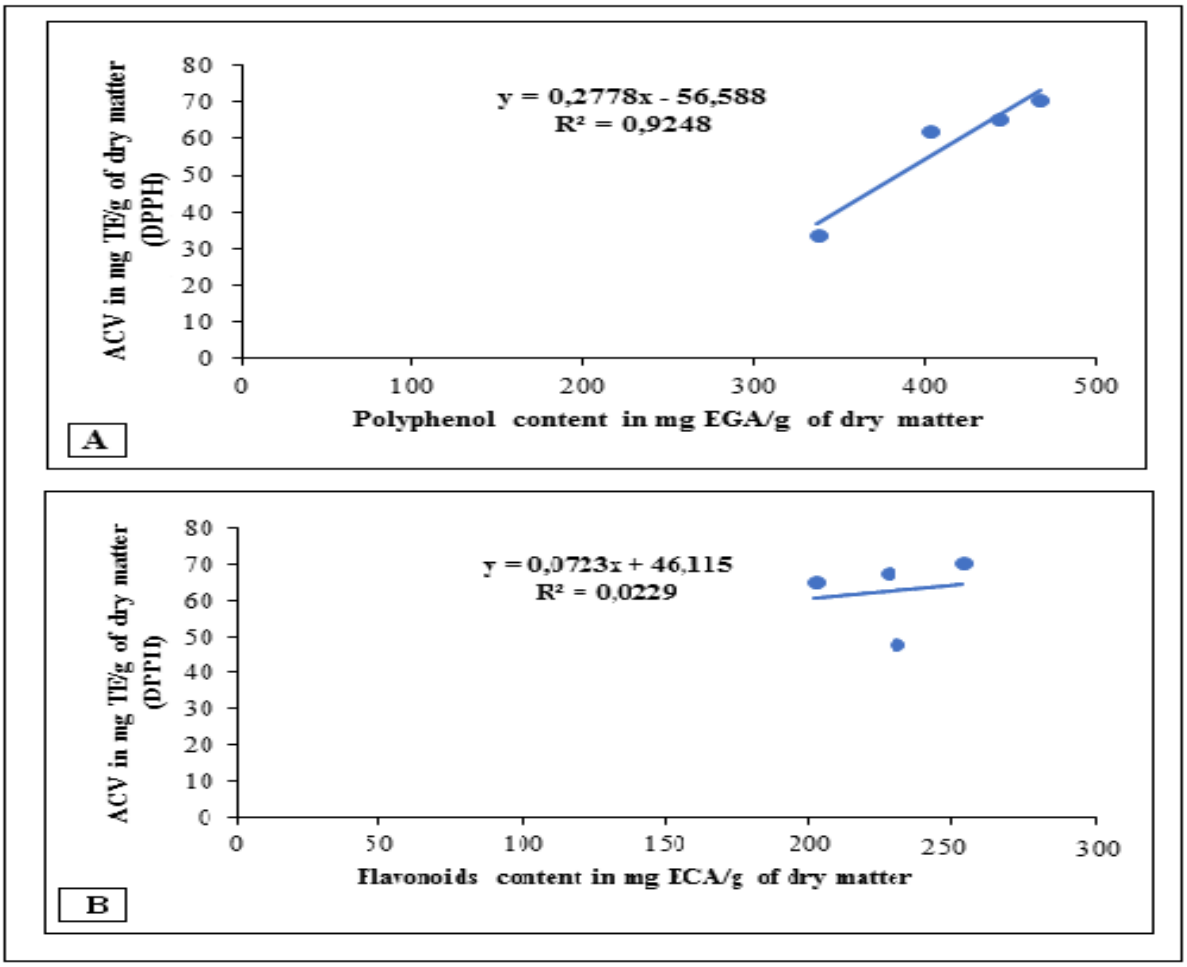

Fig. 4. Linear correlation between polyphenol (A) and flavonoid (B) content and antioxidant activity (DPPH).

The antioxidant activities of these phenolic compounds are thought to help prevent oxidative modifications by neutralizing, free radicals, trapping of oxygen or decomposing peroxides (Gonzalez-Paramas, 2018). These two active plants could be of interest in preventing diseases and reducing malaria due to oxidative stress.

Conclusion

This study highlighted the antioxidant activity of methanolic and aqueous extracts of *C. doeringii* and *D. mespiliformis*, strengthening their interest in the

treatment of malaria. The methanolic extracts of these two species studied showed the highest free radical scavenging activity and good antioxidant capacity. The high levels of total polyphenols and total flavonoids found in these plants would explain the antioxidant potential observed in the species studied, which could serve as sources of natural antioxidants needed for prevention of numerous diseases linked to oxidative stress, such as malaria.

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