Article published online: 2022-07-09

A New Colorimetric DPPH Radical Scavenging Activity Method: Comparison with Spectrophotometric Assay in Some Medicinal Plants Used in Moroccan Pharmacopoeia

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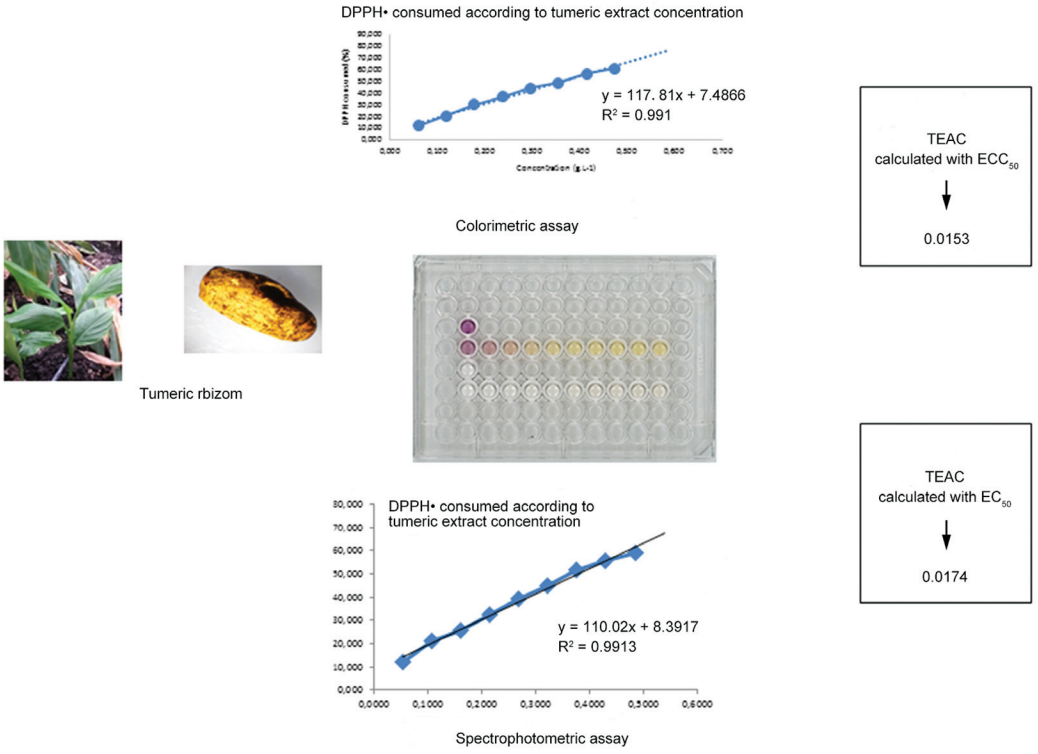
Pharmaceut Fronts 2022;4:e89–e102.



received
November 24, 2021
accepted
March 1, 2022

DOI https://doi.org/10.1055/s-0042-1748031.
ISSN 2628-5088.

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

Abstract

Antioxidants in medicinal plants are particularly important in protecting against reactive oxygen species (ROS)-related diseases, such as heart and blood vessel disease, nervous system degeneration, and cancer. Because our bodies are not strong enough to completely neutralize ROS, we sometimes need antioxidant supplementation from herbs. There is ample empirical evidence in traditional pharmacopoeias. The antioxidant activities of plant drugs have long been spectrophotometrically measured with 2,2-diphenyl-1-picrylhydrazyl (DPPH). In this study, a new colorimetry DPPH radical scavenging activity method (validated to ICH standards) for some medicinal plants, used in Moroccan pharmacopoeia, was reported and made a comparison with spectrophotometric assay. In the method, a solution of DPPH$^{\bullet}$ is incubated in the presence of an antioxidant control (Trolox) or medicinal plant extracts in wells on a 96-well plate. After an appropriate reaction time, in the dark, the plate was scanned and images obtained were processed and analyzed by Image J software. This analysis will allow us to evaluate substance's antioxidant activity, almost in the same way as a spectrophotometric assay. The colorimetric DPPH$^{\bullet}$ method exhibited a strong correlation ($R^2 > 0.95$) with the conventional spectrophotometric DPPH$^{\bullet}$ method. The colorimetric DPPH$^{\bullet}$ method had excellent accuracy (103.81–105.47%), precision (1.051–10.85% RSD [relative standard deviation]), reproducibility (1.457% RSD), and robustness (1.05–1.38 F test). The developed DPPH$^{\bullet}$ test was easy, fast, low cost and reliable, and can be used for high-throughput assay for screening DPP-scavenging activity in herb medicines.

Keywords

- colorimetric DPPH$^{\bullet}$ method
- herbal drugs
- antioxidant activity
- screening
- pharmacopoeia

Introduction

There is a wealth of experience, accumulated over the centuries, concerning herbal preparations' uses for preservation of health. In addition, data from modern scientific research on herbal drugs discovery are widely available.[1] Pathologies related to oxidative stress such as gene expression abnormalities, cell death and apoptosis, and immune disorders are increasingly common in men on a daily basis. Plant antioxidants have demonstrated in the scientific literature their enormous potential to reduce cardiovascular risk, prevent cancer, protect eyes, fight pollution, and especially delay effects of aging. Given above, the studying the antioxidant potential of plants is of great importance today. Antioxidants are known to suppress the radical generation reaction (ABTS and 2,2-diphenyl-1-picrylhydrazyl [DPPH$^{\bullet}$]) by giving electrons and inhibiting colored radicals' formation. DPPH$^{\bullet}$ scavenging assay is an electron transfer-based assay and is commonly used to assess antioxidant capacity of herb plants. It can provide reliable results under different DPPH$^{\bullet}$ concentrations, organic solvents, antioxidant(s)/free radical volume ratios, and the reaction times. Mechanically, antioxidants react with a colored probe (*oxidizing agent* DPPH$^{\bullet}$), and the color change is determined by measuring absorbance (at 515–517 nm) with a spectrophotometer. The decrease of radical DPPH absorbance is related to the degree of color development and the concentration of antioxidant metabolites in the extract. When DPPH$^{\bullet}$ reacts with antioxidant extracts, its color changes from deep purple to yellow, and the kind of discoloration makes it possible to measure the antioxidant efficiency.[2]

Although this method is considered to be very simple and effective, it has various limitations, and one important limit was the requirement of a spectrophotometer (the major disadvantage) and the consumption a lot of chemicals, and thus significantly raising the costs. To complement our validated and recently published spectrophotometric method for DPPH$^{\bullet}$ assay,[3] we hope to propose a validated preliminary phytochemical screening protocol that can be used "routinely" to improve our opportunities for discovery of new bioactive plants. In 2017, Akar et al reported a novel colorimetric DPPH$^{\bullet}$ scavenging activity method without the use of a spectrophotometer. In Akar et al's method, the mixtures of solutions of DPPH$^{\bullet}$ and standard antioxidants (or extract from common antioxidant medicinal herbs) were dropped onto thin layer chromatography (TLC) plates, and then incubated at indicated time.[4] The spot images were evaluated with Image J software (free downloadable color measurement software) to determine the CSC_{50} value. Inspired by the assay method on TLC plates or on chromatography paper, developed by Akar, we have set up a new method of assay which is done on 96-well plates. This method allows us to obtain reaction media directly exploitable by image processing software, without the disadvantages of an irregular deposit in quality and quantity on a TLC or paper. We thus avoid all artifacts related to the sampling and deposits. On the other hand, the protocol being standardized, all wells are comparable and the CCE50 obtained are directly comparable between them. Finally, the method is new since media and work protocols are totally different and not yet described elsewhere.

In our colorimetric method in this study, color measurement was performed using a smooth surface (micro-well plate) and a scanner, and images obtained by scanner were treated by Image J software. The method needs lower amounts of reagents and solvents, avoids use of the costly spectrophotometers, and affords a convenient implementation. It may represent a second complementary test method to carry out preliminary screening necessary for preselecting most bioactive plants.

As part of our extensive screening work, we present in this article antioxidant activity study of 14 plants used essentially in Moroccan pharmacopoeia and *Croton campestris*, which is interesting today because of bilharzia reappearance in France. We also proposed the practical recommendations of the developed colorimetric methods. The position of wells must be well chosen. Indeed, wells at plate periphery should not be used to avoid asymmetric surfaces. Finally, it is always necessary to ensure that the well base is well rounded to obtain the best and most reproducible measurements.

Materials and Methods

Reagent and Instrumentation

Simultaneously plant samples and Trolox standard (*same concentrations*) were studied in spectrophotometric and colorimetric DPPH methods. The radical compound DPPH$^{\bullet}$ (2,2-diphenyl-1picrylhydrazyl), Trolox, ethanol, ethyl acetate, methanol, and acetic acid were procured from VWR International S.A.S, Fontenay-Sous-Bois, France).

Instrumentations in spectroscopic DPPH analysis were VWR single beam UV-visible spectrophotometers (VWR international S.A.S, France), model UV-1600PC (VWR Collection Manual ver 1, rel. 15/05/2007) with quartz cells of 10 mm travel length. A VWR scale model LCP-423P (VWR international S.A.S, France) was used for assay with an accuracy of 0.1 mg.

Instrumentations in colorimetric DPPH analysis were plate, 96 wells, flat bottom with low evaporation lid (FALCON References: 353072, sterile R)—Tissue Culture < reacted by vacuum Gas Plasma Polystyrene Nonpyrogenic individually Packaged (Society: Corning Incorporated, One Riverfront Plaza, Corning, New York, United States) and a flatbed scanner (Canoscan, LIDE 110, Japan) in which the color photo mode was set for scanning images. The color value of each well on plate was determined as a mean gray value (MGV) by "Image J" software (the National Institutes of Health in the United States). The scanned files are saved with the following appropriate settings: contrast (85%), brightness (100), and photo mode with 600 dpi resolutions. Color change is evaluated by scanning image and using free image processing software: Image J (Free Image J program, developed by the National Institutes of Health in the United States). The images were saved as jpeg files. Color measurement was made by using a smooth surface (well of plate with 300 µL), a scanner, and the free downloadable color measurement software Image J. The color value of each well on plate was determined as MGV by "Image J" software.

Plant Tested

Artemisia annua L. Asteraceae, *Berberis hispanica* Boiss. & Reut. Berberidaceae, *Chamaerops humilis* L. Arecaceae, *Curcuma longa* L. Zingiberaceae, *Cyperus rotundus* L. Cyperaceae, *Juniperus phoenicea* L. Cupressaceae, *Lupinus albus* L. Fabaceae, *Olea oleaster* Hoffmanns. & Link Oleaceae, *Pennisetum typhoïdes* Trin. Poaceae, *Bambusa vulgaris* Schrad. ex J. C. Wendl. Poaceae, *Quercus faginea* Lam. Fagaceae, *Retama monosperma* (L.) Boiss. Fabaceae, *Ziziphus lotus* Lam. Rhamnaceae, and *Phoenix canariensis* H. Wildpret Arecaceae (part of plants used to prepare traditional medicines)[5] were purchased in Rabat from a traditional herbalist shop, Morocco, and identified by Dr. F. EL Babili *Croton campestris* (Lot: R174, code: P520FS, Rio de Janeiro, Brazil) and *Artemisia annua* (ethnobotanical spiral of Botanical Garden Henri Gaussen, 2 rue Lamarck 31400, Toulouse) was appropriately identified by Dr. F. EL Babili and Prof. I. Fouraste.

Preparation of Extracts

The fresh drugs were picked and then dried for at least a week. They were then reduced to powder by means of a mortar and a pestle (VWR international S.A.S, France). Ethanol extract of different herb medicines was separately prepared according to the following method. Dried powder of plant (10 g) was dispersed in 100 mL of ethanol, and shook continuously. The mixture was heated to 50°C for 30 minutes. After cooling down, the mixture was filtered in a funnel using Whatman No. 1 (US) filter paper. The filtrate was stored in hermetically sealed bottles in a freezer. The obtained ethanolic extract (1 mL) of the drug was diluted in 25 to 50 mL of ethanol to achieve the S1 stock solution with a standard range. The dilution volume varies depending on strength of antioxidant activity found in the preliminary test. The S1 solutions are then used to perform the standard range for each test.

Preparation of DPPH Radical Solution

DPPH$^{\bullet}$ powder (3.7 mg) was added into ethanol (25 mL). The initial concentration of DPPH$^{\bullet}$ was 375 µmol/L, which was then diluted. The absorbance values should be less than 1.0 for 50 to 100 µmol/L of DPPH$^{\bullet}$ concentration, and between 1 and 1.2 for 375 µmol/L of DPPH$^{\bullet}$ concentration.[6] The solution can only be stored for a few days (1 day in a refrigerator and several weeks in a freezer because the free-radical DPPH stock solution slowly deteriorates).

Preparation of Trolox Standards

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble derivative of vitamin E with potent antioxidant properties. In this study, Trolox was used as an antioxidant standard. Trolox (1.88 mg) was dissolved in 50 mL of ethanol. The obtained Trolox solution (150 mol/L) was diluted to obtain a series of Trolox standards (concentration range: 0.002, 0.004, 0.006, 0.008, 0.01, 0.012, 0.014, 0.016, 0.018, and 0.02 mg/mL), which were then stored in a freezer in darkness before the use.

Methods for Assessing Antioxidants' Presence in Plants

The method developed by Blois[6] to determine antioxidant activity uses the free-radical-stable α,α-diphenyl-β-

picrylhydrazyl (DPPH•; $C_{18}H_{12}N_5O_6$, M = 394.33). The assay is based on scavenging capacity measurement of antioxidants. The odd electron of the nitrogen atom in the DPPH radical is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine.[7] DPPH• used in the free-radical test is 2,2-diphenyl-1-picrylhydrazyl, a stable free radical that works in combination with other free radicals. This compound was one of the first free radicals used to study the structure/activity relationship between phenolic compounds and the antioxidant properties of herbal drugs. This is a widely used test because it is simple and relatively reliable. DPPH• is a black powder composed of stable free-radical molecules soluble in methanol or ethanol. This radical has a free electron on an atom of the nitrogen bridge. This electronic delocalization results in a characteristic blue-violet coloration of the reagent. When DPPH• reacts with an antioxidant, a hydrogen atom attaches to the radical, resulting in a loss of color. In fact, purple becomes yellow over time. The color change determined by absorbance measurement makes it possible to measure the antioxidant efficiency by spectrophotometry between 515 and 518 nm. Consumption of DPPH• was estimated based on absorbance difference between the absence (A_0) and the presence of antioxidant compounds (A_1) after a specified period (40–50 minutes). The DPPH• method has been widely applied to estimate antioxidant activity, but its applications should be performed with the basis of method in mind and the need, if possible, to establish the stoichiometry of reaction. Only then can antioxidant activity be linked to the antioxidant compounds present in the plant extract studied.

Conventional Spectrophotometric DPPH Method

Blois's DPPH• scavenging assay is a simple and relatively reliable test to determine the antioxidant properties of herbal drugs.[6] The scavenging of the odd electron of nitrogen atom in DPPH is based on receiving a hydrogen atom from antioxidants to the corresponding hydrazine, resulting in a loss of color of the reaction mixture.[7] Various concentrations of extract of the sample (mL)/Trolox were mixed with methanolic solution containing DPPH (375 µmol/L, mL). The mixture was shaken vigorously and left to stand for a specified period (40–50 minutes) at room temperature. The absorbance was measured by UV-Vis spectrophotometry at 515 nm. Regent blank and solvent control tests were also performed. The absorbance reduction measurement results can be represented as the efficient concentration to reduce 50% of DPPH•, also called EC_{50}. The DPPH• consumed is calculated by **Eqn. (1)**:

$$\text{DPPH}^{\bullet}{}_{\text{consumed}}\,\% = 1 - \left[\frac{\text{Abs.extract}}{\text{Abs.control}} \times 100\right] \qquad (1)$$

Where Abs.control is absorbance of control and Abs. extract is absorbance of test (extract)/Trolox.

The antioxidant activity of the extracts was expressed as EC_{50}, which is defined as extract concentration required to cause a 50% decrease in initial DPPH• concentration, and was calculated from a standard calibration curve ($y = ax + b$) drawn using the Microsoft Office Excel program. Trolox was used as a standard at 0.002–0.02 mg/mL. All measurements were performed in quintuplicate or sextuplicate. Lower EC_{50} values indicate higher radical scavenging potential.

Colorimetric DPPH Method

The incubation of the reaction medium (extracts + DPPH solution) was done as in the spectrophotometric method. The reaction medium was shaken for 30 seconds, and then placed in micro-wells in the dark for 40 to 60 minutes. Then plates were scanned by a Multifunctional Digital systems Model-DP eStudio 2518A Toshiba scanner. Scanned files are saved with appropriate color settings (contrast 85%, brightness 100) with 600 dpi resolution. Color value of each spot on TLC plates was measured as MGV by Image J software. The MGV grow corresponds to DPPH• concentration decrease. This reduction measurement can be represented as a percentage ECC_{50}. The DPPH• consumed is calculated by **Eqn. (2)**:

$$\text{DPPH}^{\bullet}{}_{\text{consumed}}\,\% = 100 - \left[\frac{(\text{MGV1} - \text{MGV2})}{(\text{MGV3} - \text{MGV4})} \times 100\%\right] \qquad (2)$$

With

- MGV1 corresponds to sample without DPPH•.[8] This factor is interesting because it makes it possible to eliminate any possibility of artifacts in assay which could be due to extract constituent, in particular colored.
- MGV2 corresponds to the sample in the presence of DPPH•.
- MGV3 corresponds to ethanol (*solvent used for extracts*).
- MGV4 corresponds to DPPH• solution.

To eliminate influence of initial coloring of each extract, the MGV value of the reaction medium is corrected by an appropriate subtraction.[9,10] Color intensity of the reaction medium (MGV delta = MGV1–MGV2) was obtained by subtracting color intensity of the sample from that of reaction medium, without sample. ECC_{50} (g/L) is defined as the extract concentration required to cause a 50% decrease in the initial concentration of DPPH radicals when reduced by an antioxidant, and calculated from the standard calibration curve ($y = ax + b$) analyzed using the Microsoft Office Excel program. Trolox was used as a standard at 0.0024–0.024 mg/mL. All measurements were done in quintuplicate or sextuplicate.

Colorimetry in TLC Assays

The mobile phase for all plants extracts was ethyl acetate/methanol/water (80/19/5, V/V/V), except for *Quercus faginea* and *Berberis hispanica* (ethyl acetate/acetic acid/water: 7/2/2, V/V/V). The development of TLC is carried out in a chromatographic tank with a mobile phase over a distance of 10 cm. The TLC are then dried by evaporation of the solvents under an extractor hood. The dried TLCs are completely soaked with DPPH reagent. After drying, the TLCs are scanned under the same conditions as micro-well plates. Herein, two techniques were used to deal with the TLC photos by image J software. The first is to keep the "RGB color" type for the image obtained after having scanned it (*exactly in the same parameters as for the colorimetry*). The

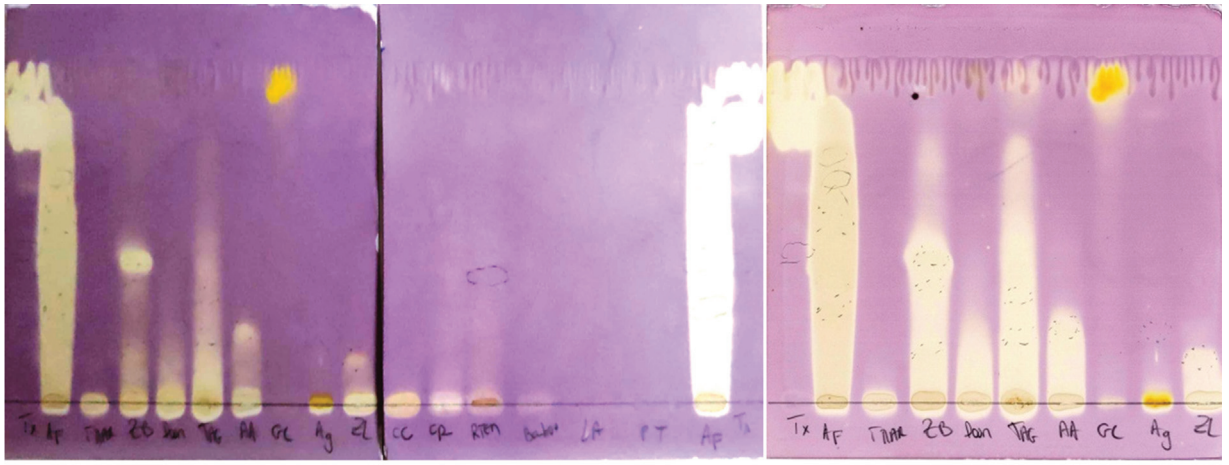


Fig. 1 TLC showing drugs sorted in descending order according to their TEAC with 5 µL (TLC 1) and 2.5 µL (TLC 2) deposits. The TLC reveals to the DPPH allows a qualitative evaluation when working in "RGB color" type: AF (*Quercus faginea)*; Tmar (*Phoenix canariensis*); Zbz (*Olea oleaster*); DOUM (*Chamaerops humilis*); TAG (*Juniperus phoenicea*); AA (*Artemisia annua*); CL (*Curcuma longa)*; Ag (*Berberis hispanica*), ZL (*Ziziphus lotus*); CC (*Croton campestris*); CR (*Cyperus rotundus*); RTEM (*Retama monosperma*); B (*Bambusa vulgaris*); LA (*Lupinus albus*); PT (*Pennisetum typhoïdes*); Tx (Trolox)/mobile phase used (ethyl acetate/methanol/water: 80/19/5 [V/V/V]). TEAC, Trolox equivalent antioxidant capacity; TLC, thin-layer chromatography.

analysis of TLC 1 (►**Fig. 1**), by software Image J, allows the generation of a profile with tool "plot lanes" (►**Fig. 2**) which will then be used with the following tools. First, we delineate the peaks using the "linear tool," and then, using the "magic wand," we obtain the measure of these peaks. To do this, we moved to the "gels" toolbar and then to the "peak labels," which allowed us to generate a table of area measurements and find each percentage of peaks. This semiquantitative analysis, although not very precise, nevertheless allows a very interesting preliminary qualitative analysis. The second technique used is to work on the photo transformed into "8 bits" (►**Fig. 3**) (*we end up in black and white from 0 to 255 pixels*). In this case, we work by rectangular lane (*select the first lane then the second and do the same for the following ones*), each corresponding to a drug. Once all lanes have been created, all that remains is to measure the MGV corresponding to each lane with the tools "analyze" and "measure."

Calculation of Trolox Equivalent Antioxidant Capacity

The DPPH radical scavenging activity of sample was expressed as Trolox equivalent antioxidant capacity (TEAC). TEAC was calculated as **Eqn (3)**[11]:

$$\text{TEAC} = \frac{\text{EC}_{50} \text{ or } \text{ECC}_{50} \text{ of Trolox (g/L)}}{\text{EC}_{50} \text{ or } \text{ECC}_{50} \text{ of Sample(g/L)}} \times 1000 \text{ (mg/L)} \qquad (3)$$

EC_{50} or ECC_{50} values of Trolox/sample should be measured on the same day. The higher TEAC value means the higher DPPH radical scavenging activity.

Validation (Precision, Accuracy, Reproducibility, and Robustness) and Statistical Analysis

Data were expressed as means ± standard deviations of triplicate measurements. The curves for EC_{50} or ECC_{50} value calculations were constructed by running standards or plant extracts of five different concentrations, in triplicate. Trolox

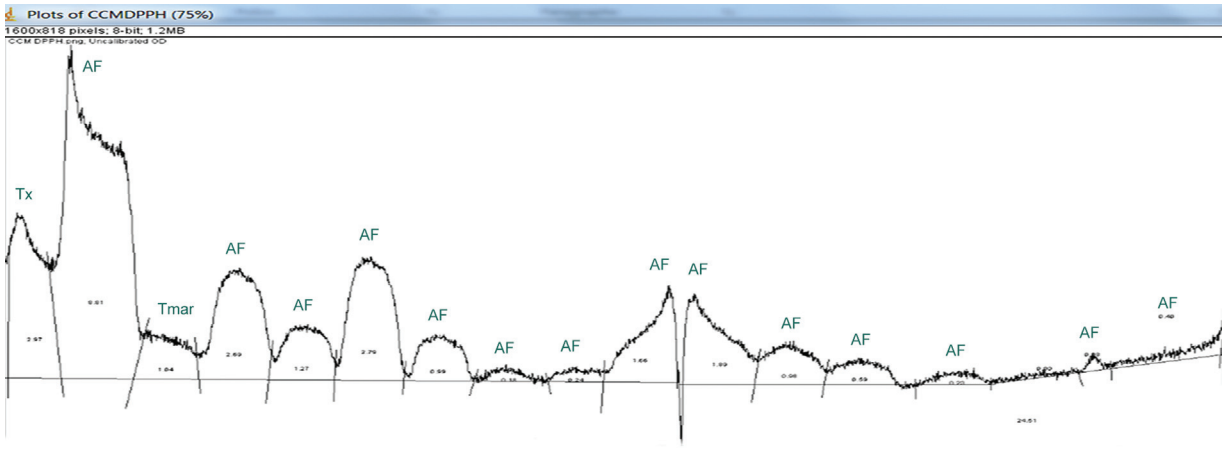


Fig. 2 Plot lanes with peak label in image J with type "RGB color.

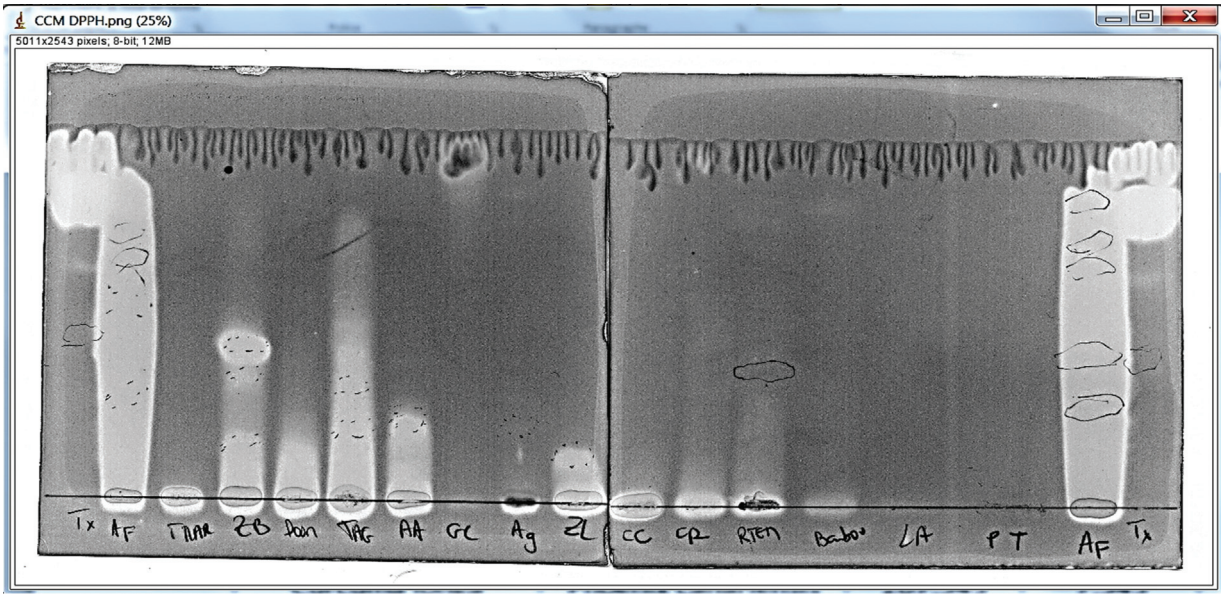


Fig. 3 Profile in image J with type "8-bit."

was used as the standard for the DPPH assays. The similarity for 50% DPPH$^{\bullet}$ scavenging concentrations of the standards and samples between the conventional method and the new method was determined with an R^2 value >0.95. The parameters' validation, including linearity, limits of detection, precision (repeatability, intermediate precision, and reproducibility), accuracy, and robustness, was performed considering Q2 (R1) guidelines.[3,11,12]

Results

Analytical ICH Method Validation Parameters

The developed method was validated in accordance with ICH (International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use) guidelines with Trolox (Q2) (R1).[12]

Linearity

The calibration curve consisted of 10 different concentrations in the range of 0.0008 to 0.008 mg/mL for Trolox solution. The regression equation gives a correlation coefficient superior to 0.99 between standard concentration (x) and mean absorbance ($n = 5$), showing a good linearity of the standard curve. The calibration curve with the regression equation was $y = 14635x + 0.846$ with a good correlation coefficient (0.996). The curve was obtained by plotting mean DPPH$^{\bullet}$ ($n = 5$) consumed % (y) based on analyte (x) concentration in mg per mL.

Precision

Repeatability

The relative standard deviation (RSD) is the variation in the coefficient absolute value. It is generally expressed as percentage. RSD is equal to standard deviation in relation to the average and multiplied by 100. The variation coefficient was taken as repeatability measures. Since the variation coefficient is always less than or equal to 10%, the determination method provides consistent results (►**Table 1**). The method is precise.

Intermediate Fidelity: Dosages Performed from One Day to the Next

The equal variance test (F-test) was taken as a measure of the intermediate precision. Statistical comparisons of DPPH$^{\bullet}$ consumed % as a function of antioxidant concentration reveal that there are no significant differences in UV assays of antioxidant capacity from day to day in Trolox standard. The means of DPPH$^{\bullet}$ consumed % are not significantly different (►**Table 2**) because in F-test the $F_{\text{calculated}}$ is always inferior to F_{critical}.

Reproducibility

The % RSD for tests with different devices' (S-JBHG and S-LGC) values for intra-assay precision and intermediate precision for concentration level of 0.025 were below 2% (►**Table 3**), indicating the developed method's good reproducibility.

Accuracy

Trolox accuracy is obtained by recoveries between 103.81 and 105.47% (►**Table 4**). The results obtained support the developed method's precision. The accuracy was reported as % recovery ± standard deviation. Accuracy values obtained were in the range of 95 to 110%.

Table 1 Results of the repeatability study of Trolox

Studied samples	% RSD for inter-day tests	Average % of RSD
Trolox	10.85	2.56

Table 2 Results of intermediate fidelity study of Trolox and plants studied

Tested samples Intraday ($n = 2$)	DPPH• consumed average % in J0	DPPH• consumed average % in J1	$F_{calculated}$	F-test $< (F_{critical})$
Trolox	55.68	58.53	1.051	3.179

Table 3 Results of intermediate fidelity study of Trolox and plants studied

Tested samples	DPPH• consumed % average in JBHG	DPPH• consumed % average in LGC	% RSD (variation coefficient) for tests with different devices
	24.312	26.372	1.457

Table 4 Accuracy results for Trolox standard and plants studied

Extract studied	Concentrations (mg/L)	Theoretical value in % of test sample	% DPPH• consumed measured	% DPPH• consumed recalculated	% Recovery[a] (mean ± SD)
Trolox	0.0096	80	48.77	46.24	105.47
	0.0120	100	54.87	52.85	103.81
	0.0144	120	62.36	59.47	104.85

Abbreviation: SD, standard deviation.
[a]Indicates mean of five determinations ($n = 5$).

Table 5 Robustness results (different equipment) for Trolox standard

Plant drug extracts studied	Average consumed DPPH• %—S-JBHG	Average consumed DPPH• %—S-LGC	F-test $F_{calculated}$ and $F_{critical}$
Trolox	55.68	59.67	$1.38 < 3.18$

Table 6 Robustness results (different analyst) for Trolox standard

Plant drug extracts studied	Average consumed % DPPH•—analyst A	Average consumed % DPPH•—analyst J	F-test $F_{calculated}$ and $F_{critical}$
Trolox	55.68	58.53	$1.05 < 3.18$

The results show that recoveries obtained from the 100% standard are between 95 and 110%. The method is therefore accurate in the field tested: at 0.0096, 0.0120, and 0.0144 mg/L for Trolox. The obtained results support the developed method's accuracy.

Specificity

The developed method was found to be selective and specific as there was no interference occurred as reflected by accuracy results.

Robustness

Variations in stability of colorimetric assay, subject to the use of different equipment and intervention of two different analysts, gave good results (►**Tables 5** and **6**), indicating robustness of the method proven today.

Conclusion on ICH Validation: New Colorimetric Method

The developed method was successfully applied to determine antioxidant properties. In accordance with ICH guidelines, assay values for all types of samples studied were found to be within standards. The linearity, accuracy, and recovery rate of colorimetric method used to evaluate antioxidant activity of Trolox are validated. The results indicate a good suitability of the assay method for evaluation of antioxidants. This method can now be used routinely for phytochemical screening. Under optimal conditions, the calibration curve showed a good linear regression (►**Fig. 4**) and reproducibility (►**Table 4**). The overall intra-day and inter-day variations are less than 10%. Finally, recovery tests of consumed DPPH• in % shown in ►**Table 4** gave results between 95 and 110%, as expected according to ICH standards.

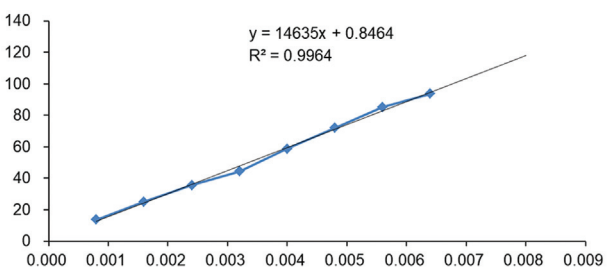


Fig. 4 % DPPH• consumed based on Trolox concentrations.

Table 7 Results of antioxidant activities of 15 medicinal plants by two method assays: spectrophotometry and colorimetry

Ethanolic extracts of tested Plants	Spectrophotometric DPPH assay (EC_{50} [g/L])	Colorimetry DPPH assay (ECC_{50} [g/L])
Moroccan pharmacopoeia plants		
Berberis hispanica Boiss. & Reut. Berberidaceae	0.474–$R^2 = 0.96$	0.436–$R^2 = 0.99$
Chamaerops humilis L. Arecaceae	0.21–$R^2 = 0.99$	0.208–$R^2 = 0.99$
Curcuma longa L. Zingiberaceae	0.386–$R^2 = 0.99$	0.458–$R^2 = 0.989$
Cyperus rotundus L. Cyperaceae	0.612–$R^2 = 0.97$	0.5955–$R^2 = 0.99$
Juniperus phoenicea L. Cupressaceae	0.2238–$R^2 = 0.99$	0.211–$R^2 = 0.99$
Lupinus albus L. Fabaceae	24.54–$R^2 = 0.989$	28.58–$R^2 = 0.99$
Olea oleaster Hoffmanns. & Link Oleaceae	0.188–$R^2 = 0.98$	0.235–$R^2 = 0.99$
Pennisetum typhoides Trin. Poaceae	7.22–$R^2 = 0.98$	6.48–$R^2 = 0.99$
Bambusa vulgaris Schrad. ex J. C. Wendl. Poaceae	4.636–$R^2 = 0.96$	4.99–$R^2 = 0.99$
Quercus faginea Lam. Fagaceae	**0.0072** – $R^2 = 0.96$	**0.0078**–$R^2 = 0.988$
Retama monosperma (L.) Boiss. Fabaceae	1.38–$R^2 = 0.99$	1.77–$R^2 = 0.99$
Ziziphus lotus Lam. Rhamnaceae	0.5962–$R^2 = 0.90$	0.772–$R^2 = 0.987$
Phoenix canariensis H.Wildpret Arecaceae	0.1177–$R^2 = 0.99$	0.159–$R^2 = 0.99$
Chinese pharmacopoeia plant		
Artemisia annua L. Asteraceae (International Plant Names Index, n.d.)	0.368–$R^2 = 0.90$	0.3352–$R^2 = 0.99$
Brazilian Pharmacopoeia Plant		
Croton campestris st Hil. Euphorbiaceae	0.611–$R^2 = 0.955$	0.806–$R^2 = 0.93$
Standard		
Trolox	0.0067–$R^2 = 0.987$	**0.0070**–$R^2 = 0.99$

Spectrophotometric and Colorimetric Assay Results

As shown in ►**Table 7**, the ECC_{50} (g/L) value of the colorimetric DPPH method is very similar to the EC_{50} values of the spectrophotometric DPPH method. The similarity for 50% $DPPH^{\bullet}$ scavenging concentrations of the standards and samples between the conventional method and the new method was determined with an R^2 value >0.95 (see most often with an $R^2 > 0.98$). The antioxidant capacities, expressed in TEAC, followed a hierarchic order (*from the most active drug to the least active compared with Trolox control*) (►**Table 8**): *Quercus faginea* (0.93–0.89), *Phoenix canariensis* (0.057–0.044), *Olea oleaster* (0.036–0.030), *Chamaerops humilis* (0.032–0.034), *Juniperus phoenicea* (0.030–0.033), *Artemisia annua* (0.018–0.021), *Curcuma longa* (0.017–0.015), *Berberis hispanica* (0.014–0.016), *Ziziphus lotus* (0.011–0.009), *Croton campestris* (0.011–0.009), *Cyperus rotundus* (0.011–0.012), *Retama monosperma* (0.005–0.004), *Bambusa vulgaris* (0.001–0.001), *Pennisetum typhoïdes* (0.001–0.001), and *Lupinus albus* (0.0003–0.0002). TLC analyses are shown in ►**Figs. 1**,**2**,**3** and in ►**Tables 9**,**10**,**11**. For the colorimetric $DPPH^{\bullet}$ method, the comparison of TLC results by image J and TEAC values showed that there is a slight change in classification of drugs according to their antioxidant power, for only three drugs (►**Table 9**). However, in general, there is a very good correlation. There are still technical difficulties in TLC methods in colorimetric DPPH assay, for example saturation when deposits are too concentrated. These deposits must be better calibrated and individualized according to the properties of each drug so that the calculation can possibly be corrected according to the concentration deposited. It is precisely this problem of concentration of deposits that explains the small differences found in our results. ►**Table 10** demonstrates the results comparing TEACs with TLC colorimetric assays operated fewer than two protocols, i.e., "RGB color" and/or "8 bit." There are differences between the "RGB type," or where the peaks are evaluated while in the "8-bit type," it is the MGV that is measured (*more accurate difference with control value without DPPH discoloration*). These differences are used to classify plant drugs according to their antioxidant power. Although not giving exactly the same classification, the colorimetric technique applied on TLC nevertheless makes it possible to identify the drugs located at the extremes, that is to say the most active and the least active. As explained above, the technique by TLC still deserves some adjustments. For example, the initial colorations of plant extracts and their concentrations must be better taken into account so that this method is eventually used in a completely reliable way, or even independently of the assays. The work is ongoing.

Discussion

To be able to interpret the obtained results, we worked from a state of knowledge on the 15 plants of our studies, which allowed us to draw the relevant conclusions for each drug.

Table 8 Classification of drugs according to their antioxidant capacity in Trolox equivalent

	EC_{50} (g/L)	ECC_{50} (g/L)	TEAC calculated with EC_{50}	TEAC calculated with ECC_{50}	Decreasing ranking in antioxidant strength
Trolox	**0.0067**	**0.007**	1.0000	1.0000	**1** (most active)
Quercus faginea Lam. Fagaceae	**0.0072**	**0.0078**	**0.9306**	**0.8974**	**1**
Phoenix canariensis H. Wildpret Arecaceae	0.1177	0.159	0.0569	0.0440	**2**
Olea oleaster Hoffmanns. & Link Oleaceae	0.188	0.235	0.0356	0.0298	**3**
Chamaerops humilis L. Arecaceae	0.21	0.208	0.0319	0.0337	**4**
Juniperus phoenicea L. Cupressaceae	0.2238	0.211	0.0299	0.0332	**5**
Artemisia annua L. Asteraceae	0.368	0.3352	0.0182	0.0209	**6**
Curcuma longa L. Zingiberaceae	0.386	0.458	0.0174	0.0153	**7**
Berberis hispanica Boiss. & Reut. Berberidaceae	0.474	0.436	0.0141	0.0161	**8**
Ziziphus lotus Lam. Rhamnaceae	0.5962	0.772	0.0112	0.0091	**9**
Croton campestris st Hil. Euphorbiaceae	0.611	0.806	0.0110	0.0087	**10**
Cyperus rotundus L. Cyperaceae	0.612	0.5955	0.0109	0.0118	**11**
Retama monosperma (L.) Boiss. Fabaceae	1.38	1.77	0.0049	0.0040	**12**
Bambusa vulgaris Schrad. ex J. C. Wendl. Poaceae	4.636	4.99	0.0014	0.0014	**13**
Pennisetum typhoides Trin. Poaceae	7.22	6.48	0.0009	0.0011	**14**
Lupinus albus L. Fabaceae	24.54	28.58	0.0003	0.0002	**15** (least active)

Table 9 Phytochemical study by TLC of 15 medicinal plants

Tested plants ethanolic extracts	Reagent used- qualitative phytochemical screening (intensity expressed by crosses in brackets) – R_f (retardation factor)[1] – reaction colors
Moroccan Pharmacopoeia Plants	
Artemisia annua L. Asteraceae (International Plant Names Index," 1/2 n.d.)	• D (slight trace) • NEU (++) – (0, 0.25) – fluo green • DPPH (+++) – (0, 0.23, streaks) • AP (+) – (0.9) • KOH (+) – (0, 0.7) – yellowish brown spots phenols compounds • $FeCl_3$ (++) – (0, 0.3)
Berberis hispanica Boiss. & Reut. Berberidaceae	• D (++) – (0) – brown red • NEU (++) – (0, 0.2) – green yellow fluo flavonoids • DPPH (++) – (0) • AP (++) – (0.15, 0.9) – yellow and blackish green • KOH (+++) – (0, 0.2, 0.7) – yellowish brown to red – phenols compounds and fluo (at 350 nm) flavonoids • $FeCl_3$ (+) – (0) blackish green
Chamaerops humilis L. Arecaceae	• D (−) • NEU (−) • DPPH (+++) – (0, 0.18, streaks) • AP (++) – (0.25, 0.33) • KOH (++) – (brown streaks) • $FeCl_3$ (++) – (0)
Curcuma longa L. Zingiberaceae	• D (−) • NEU (+) – (0.95) – red • DPPH (+) – (0.95) • AP (−) • KOH (+) – (0.9) • $FeCl_3$ (−) – yellow zone becoming brown

(*Continued*)

Table 9 (*Continued*)

Cyperus rotundus L. Cyperaceae	• D (−) • Neu (−) • DPPH (++) – (0) • E (++) – (0.1, 0.90) – pink spots terpenes • AP (−) • KOH (−) • $FeCl_3$ (++) – (0) – blackish green catechetical tannins
Juniperus phoenicea L. Cupressaceae	• D (+) – (0) • NEU (−) • DPPH (+++) – (0, 0.5, streaks) • AP (++) – (0, 0.39) • KOH (++) – (0 to 0.1, streaks brown) and (+) – (0.7) • $FeCl_3$ (+++) – (0)
Lupinus albus L. Fabaceae	• D (trace) • NEU (++) – (0) • DPPH (−) • AP (+) – (0.27, streaks) • KOH (−) • $FeCl_3$ (−)
Olea oleaster Hoffmanns. & Link Oleaceae	• D (−) • NEU (−) • DPPH (+++) – (0, 0.2, 0.5, streaks) • AP (−) • KOH (++) – (0.7) • $FeCl_3$ (++) – (0, 0.05, streaks) – greyish green
Pennisetum typhoides Trin. Poaceae	• D (blackish trace) – (0) • NEU (−) • DPPH (−) • AP (−) • KOH (−) • $FeCl_3$ (−)
Bambusa vulgaris Schrad. ex J. C. Wendl. Poaceae	• D (−) • NEU (trace) – (0) • DPPH (+) – (0) • AP (++) – (0.39, 0.7) • KOH [(trace) – (0), (+) – (0.4) – yellow fluo] – flavonoid • $FeCl_3$ (trace) – (0)
Quercus faginea Lam. Fagaceae	• D (−) • NEU (++++) – (0.7, 0.9 streaks) – Fluo blue phenols • DPPH (++++++) – (0 et 0.5, 0.8, 0.9, streaks) • AP (++++) – (0.5, 0.7, 0.9 streaks) • KOH (++++) – (0.5, 0.7 streaks, 0.9 fluo) – phenols flavonic heteroside and flavonoids • $FeCl_3$ (++++) – (0.5, 0.7, 0.9 streaks) – blackish tannins • E (+) – (0.95)
Retama monosperma (L.) Boiss. Fabaceae	• D (+++) – (0) • NEU (++) – (0, 0.4, 0.9) • DPPH (++) – (0, streaks) • AP (+++) – (0.25, 0.39, streaks) • KOH (++) – (0, 0.7) – brown spots • $FeCl_3$ (+) – (trace, 0, 0.4)
Ziziphus lotus Lam. Rhamnaceae	• D (−) • DPPH (++) – (0, 0.1, streaks) • AP (+) – (0.78) • KOH (+) – (0) – brown spot • $FeCl_3$ (++) – (0, 0.1) – greyish green
Phoenix canariensis H. Wildpret Arecaceae	• D (−) • NEU (−) • DPPH (++) – (0) • AP (−) • KOH (+++) – (0) – brown spot • $FeCl_3$ (++) – (0)

Table 9 (*Continued*)

Croton campestris st Hil. Euphorbiaceae	• D (+) – (0) • NEU (−) • DPPH (++) – (0) • KOH (+) – (0) – fluo – flavonoids • AP (−) • $FeCl_3$ (−)
Standard	
Trolox	• D (−) • NEU (−) • DPPH (+++++) – (0.95) • AP (++++) – (0.94) • $FeCl_3$ (−)

Abbreviations: D, Dragendoorf reagent, E, erlich reagent, DPPH, DPPH reagent, Neu, Neu reagent (TLC observed at 350 nm), AP, phosphomolybdic acid reagent (with heating), P, phloroglucinol reagent, KOH, potassium hydroxide reagent, $FeCl_3$ = Iron (III) chloride reagent, AE, ethyl acetate, Ac. A., acetic acid, MeOH, methanol.

[1]R_f was calculated from distance ratio from midpoint of spot to starting point/distance from the solvent front to the starting point.

Mobile phases for all herb drugs (except for *Quercus faginea* and *Berberis hispanica*) were AE/MeOH/H_2O (80/19/5–V/V/V), and Mobile phases for *Quercus faginea* and *Berberis hispanica* were AE/Ac. A./H_2O (7/2/2).

Artemisia annua TEAC was found to be[13–15] quite similar with our result for ethanolic extract (18.2) and methanolic extract.[13,16] Recent works (***antioxidant but also antifungal properties***) showed elements of justification for traditional uses.[17] In recent years, the use of herbal medicine for cancer therapy has been paid special attention. *Berberis hispanica* revealed high antioxidant activity and potent activity toward cancer.[18–20] Its main traditional use in Rabat is as an anticancer agent (original work not yet published by F. EL Babili and V. M. Lamade). The *Curcuma* rhizome traditionally used exhibit antioxidant properties which might be due to high amounts of phenolic compounds.[21] *Cyperus rotundus*

Table 10 Study by TLC of 15 medicinal plants

Drugs classification of according to TLC analysis by image J	Area	% peaks label	Drug classification according to TEAC	Decreasing ranking in antioxidant strength
Quercus faginea Lam. Fagaceae	43,001	8.81	*Quercus faginea* Lam. Fagaceae	1
Trolox	14,491	2.97	Trolox	1 *(most active)*
Olea oleaster Hoffmanns. & Link Oleaceae	13,633	2.79	*Olea oleaster* Hoffmanns. & Link Oleaceae	3
Chamaerops humilis L. Arecaceae	13,149	2.69	*Chamaerops humilis* L. Arecaceae	*4*
Croton campestris st Hil. Euphorbiaceae	9,210	1.89	*Croton campestris* st Hil. Euphorbiaceae	10
Ziziphus lotus Lam. Rhamnaceae	8,101	1,66	*Ziziphus lotus* Lam. Rhamnaceae	9
Juniperus phoenicea L. Cupressaceae	6,187	1.27	*Juniperus phoenicea* L. Cupressaceae	5
Phoenix canariensis H. Wildpret Arecaceae	5,089	1,04	*Phoenix canariensis** H. Wildpret Arecaceae	2
Artemisia annua L. Asteraceae	4,815	0.99	*Artemisia annua* L. Asteraceae	6
Cyperus rotundus L. Cyperaceae	4,792	0.98	*Cyperus rotundus* L. Cyperaceae	11
*Retama monosperma** (L.) Boiss. Fabaceae	2,872	0,59	*Retama monosperma** (L.) Boiss. Fabaceae	12
*Curcuma longa** L. Zingiberaceae	1,121	0.24	*Curcuma longa** L. Zingiberaceae	7
Bambusa vulgaris Schrad. ex J. C. Wendl. Poaceae	1,103	0.23	*Bambusa vulgaris* Schrad. ex J. C. Wendl. Poaceae	13
Berberis hispanica Boiss. & Reut. Berberidaceae	835	0.18	*Berberis hispanica* Boiss. & Reut. Berberidaceae	8
Lupinus albus L. Fabaceae	49	0.11	*Pennisetum typhoides* Trin. Poaceae	14
Pennisetum typhoides Trin. Poaceae	393	0.08	*Lupinus albus* L. Fabaceae	15 *(least active)*

Table 11 Drug classification (according to their AO strength) by a comparative study, between their EC_{50}, on the one hand, and their TLC treatment results per image J

Classification according to AO power according to EC_{50}	Classification according to AO power according to TLC type "RGB color"	Classification according to AO power according to TLC type "8-bit"	Mean MGV[a]
Trolox	*Quercus faginea*	Quercus faginea	233.457
Quercus faginea	Trolox	*Trolox*	223.099
Phoenix canariensis	*Olea oleaster*	*Juniperus phoenicea*	217.915
Olea oleaster	*Chamaerops humilis* L.	*Olea oleaster*	216.638
Chamaerops humilis	*Croton campestris*	*Croton campestris*	210.929
Juniperus phoenicea	*Ziziphus lotus*	*Ziziphus lotus*	210.501
Artemisia annua	*Juniperus phoenicea* L.	*Chamaerops humilis*	208.675
Curcuma longa	*Phoenix canariensis*	*Phoenix canariensis*	207.545
Berberis hispanica	*Artemisia annua* L.	*Artemisia annua*	206.946
Ziziphus lotus	*Cyperus rotundus*	*Cyperus rotundus*	205.941
Croton campestris	*Retama monosperma*	*Retama monosperma*	203.724
Cyperus rotundus	*Curcuma longa*	*Berberis hispanica*	201.759
Retama monosperma	*Bambusa vulgaris*	*Curcuma longa*	201.752
Bambusa vulgaris	*Berberis hispanica*	*Bambusa vulgaris*	201.15
Lupinus albus	*Lupinus albus*	*Lupinus albus*	200.902
Pennisetum typhoides	*Pennisetum typhoides*	*Pennisetum typhoides*	200.457

Abbreviations: AO, antioxidant activity; MGV, mean gray value; TLC, thin-layer chromatography.
[a]Quantitative analysis of images in ►**Fig. 3** by "Image J" software.

rhizome exhibited cytotoxic, apoptotic properties.[22–24] Its activities have supported its traditional as well as prospective uses as a valuable Ayurveda plant.[25] Ranked in the third position, *Olea oleaster* presents an interesting antioxidant activity. For *Quercus faginea*, its gallnuts are apparently much more antioxidant than cork and bark.[26] Moroccan folk medicine treats healing of skin with *Retama monosperma* and the extract form its seeds showed a presence of three subgroups of flavonoids, which correlated with the antioxidant activity of the plant.[27–29] These results corroborate very well with the traditional use, still in force today, among traditional practitioners. Our work demonstrates and confirms the powerful antioxidant activity of *Phoenix canariensis*, since it is ranked second in our ►**Table 2** just after *Q. faginea*.[30] *Croton campestris*, rich in polyphenols,[31,32] exhibiting anti-bilharzian[33,34] and antiparasitic activities,[35] is back in the news today, especially since this disease was detected again in Europe.[36] It seemed important to us to evaluate its antioxidant activity. This bibliographic review in our work shows that finally, with a thorough analysis, the borderline between traditional data, resulting from empirical practices still used, and scientific data is extremely reduced. This adequacy between tradition and science shows that it is certainly possible, in the near future, to join objectives fixed by the last resolution of the World Health Organization[37]: To set up a universal health care system by uniting traditional knowledge with scientific knowledge.

Conclusion

The results of the colorimetric method showed a strong correlation with results obtained by the spectrophotometric method. Thus, the applicability of DPPH test independently of spectrophotometer will offer advantages such as easy, fast, low cost, and reliability. The colorimetric method is therefore very interesting in the context of preliminary tests because it is both qualitative since result is seen directly with the naked eye and quantitative.

This technique allows for quantification by processing the scanned data with the free software Image J. This technique, although inexpensive, does not lose any of its effectiveness. It allows finding results comparable to spectrophotometric analyses. This new assay technique that we publish aims to allow small research teams, like ours, working on medicinal plants (*from traditional pharmacopoeias*) to be able to highlight the existence of new local plants, with local antioxidant properties, everywhere in the world. By exploiting local plants, there is no need to go to the other side of the world to find bioactive plants. Effective, local plant antioxidants are available everywhere. In a way, and in line with current environmental concerns, this method would be a breath of fresh air in the research on medicinal plants. The comparison of the results obtained by means of two types of tests of antioxidant capacity of each extract by spectrophotometry and by colorimetry shows a very good correspondence. The two methods seem almost equivalent since the EC_{50} and

ECC_{50} values are almost identical. Our method has the interest of being new in analytical development for the determination of antioxidant activities. Our colorimetric assay method is much less technically complex to implement and is perfectly suited to the preliminary screening work of biological activities that we perform in our laboratory. It is also an alternative method to the DPPH spectrophotometric assay for small laboratories with a limited budget but a large work capacity. We will use this method as the first step to perform a preliminary screening in our vast collection of several thousand medicinal plants, originating from all continents. Of course, it is recommended to confirm the results obtained by spectrophotometry, for the plants presenting an interesting activity ($EC_{50} < 1.5$) before starting a more thorough phytochemical analysis. But it is still a great possibility to save time and money as screening is long and tedious, in general.

This new colorimetric assay method, successfully developed in accordance with ICH guidelines, has been shown to be simple, sensitive, and specific for evaluation of the antioxidant activity in herbal medicines. The results demonstrate that the developed method is accurate, reproducible, and can be easily used as a quality control method. The developed method is therefore recommended to be implemented as a preliminary protocol in phytochemical screening.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors would like to thank Mrs. Meryem EL Kahlani for her participation as a guide, companion, and especially for her help during the field interviews with the herbalists, from whom we bought medicines and whose traditional uses we collected. The latter sold us the herbal drugs, explaining to us their traditional uses. We would also like to thank Mr. Mohammed Kabbaj, a bookseller with a passion for pharmacopoeia, for his valuable intervention which allowed us to get in touch with many traders, herbalists, and producers of medicinal plants, encountered during our fieldwork, especially in the region of Essaouira.

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