ICH Validation of DPPH Assay Method: Some Interesting Medicinal Drugs

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Abstract:

Background and Aim: As part of our antioxidant plants screening project, we conducted a phytochemical study on their antioxidant activity to provide a standard and reliable assessment tool. To carry out this extensive screening, in compliance with regulatory requirements in pharmaceutical field, we have undertaken validation of our DPPH assay. This validation is in accordance with ICH standards. Indeed, this validation procedure has a very broad scope. It applies to any analysis procedure for plant raw materials control.

Method: DPPH test created by Blois and adapted for our raw extracts study is carried out using a UV spectrophotometer at 516 nm, as a monitoring wavelength. The ICH standards are then studied using an appropriate extracts and samples number for relevant statistical analysis. The reference substance chosen is Trolox.

Results: To validate this method, we will quantify antioxidant properties in two plant drugs traditionally known for their property such as Argania spinosa (fruits and leaves) and Lawsonia inermis (ethanolic extracts and decoctions). Our results allow to specify and quantify these properties and to confirm traditional ancient use.

Conclusions: The method developed is therefore recommended as a quality control protocol in phytochemical screening. This last screening is always necessary before starting anticancer properties plants study. Plants are gradually regaining interest and a preliminary screening method will can be included in specific monograph. Thus these monographs could help to promote the heritage of traditional medicine; still alive and to which the WHO seeks to restore its place (latest resolution 2014-2023).

Keywords: ICH, Linearity, Antioxydant Ability, *Argania spinosa*, *Lawsonia inermis*, Trolox

1. Introduction

At origin of various degenerative disorders, such as aging, cancers (mutagenesis, carcinogenesis) and cardiovascular disorders, free radicals are inevitable products of biological degradation (Singh and Singh, 2008). Antioxidant homeostasis must be preserved to guarantee delicate redox balance necessary for good health.

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Therefore, exogenous intakes in antioxidants, especially through medicinal plants, must be judicious and adapted (Berger *et al*., 2012). Only balance between these radicals (harmful oxidants) and antioxidants ensures a correct state of health (Berger *et al.,* 2012; Levakova & Lacko-Bartosova, 2017; Yahfoufi *et al.,* 2018). Antioxidants are present in our body but are also reported by our diet, especially vegetable (Kedare and Singh, 2011). The search for natural antioxidants, existing in our traditional medicine arsenal represents an optimistic alternative in fight against pathologies caused by oxidative free radicals excess.

In this study, we aimed to describe phytochemical antioxidant properties of various herbals drugs extracts (Deng *et al.,* 2019) to validate this spectrophotometric method for first time in hydro-alcoholic extracts of *Argania spinosa* and *Lawsonia inermis*. For *Argania spinosa*, it is essentially oil extracted from fruit that has been studied in many research papers (Lizard *et al*., 2017). The anti-oxidative activity of their ethanolic extracts was determined using DPPH assays (Deng *et al*., 2019).

This study helps to demonstrate UV spectrophotometry relevance for rigorous quantification of the plant drug antioxidant properties, in its traditional form of decoction and/or hydro-alcoholic extract (Lee *et al.,* 2020). This method interests us because we have implemented a broad phytochemical screening of antioxidant capacities. We work on plant extracts derived from plant drugs still actively used in the world. Our rich university heritage collection and our JBHG druggist offer many potentially active samples. Thanks to our results of ethnobotanical surveys that are beginning to accumulate, it seems urgent to have a method validated according to the ICH standards in force.

The renewed interest in herbal medicine here gives full meaning to such screening work (Garcia, 2020). According to WHO (2013), traditional medicine will be positioned in future alongside conventional medicine so that access to care is possible everywhere in the world. The antioxidant capacity evaluation, validated according to ICH standards (Q2r1-Fra.Pdf), will be a valuable tool that will allow us to enrich our knowledge on medicinal plants, at least by discovering the most active, while opening new avenues in phytochemical research. This concern to be more nature respectful and study of plant extracts identical to those always used in traditional medicine, allows combining both phytochemistry and tradition. During COVID 19 pandemic, there was a definite return to traditions both in countries where modern medicine remains unaffordable and in rich countries such as France (Lectoure) (El Babili *et al.,* 2020). The method validation for evaluating antioxidant capacities, used as control Trolox (Blois, 1958) and as extract medicinal plants recognized as *Argania spinosa*, *Lawsonia inermis*, among others. It offers a simple, accurate and validated method. All aspects considered in validation procedure, in accordance with ICH guidelines, have been complied with those related exception to stability, as this work is ongoing.

The method will be evaluated using ICH guidelines for linearity, specificity, sensitivity, accuracy, and robustness. Stability is a study that covers six months to three years. Indeed, a phytodrug has a fairly limited shelf-life (2 to 3 years at most), according to its stability data (expiry). On the other hand, plant drugs remain, according to tradipraticians, usable much longer. There is stability data defined according to standards such as Trolox. But the expiry date of plant drugs remains an important concept to define for better use tomorrow.

2. Materials and Methods

2.1 Plant Material

2.1.1 Instrumentation

The spectroscopic analysis was performed using VWR single beam UV-visible spectrophotometer, model UV-1600PC, with quartz cells of 10 mm travel length. The solutions were carried out on dry drugs based on mass, using a VWR scale model LCP-423P with an accuracy of 0.1 mg.

2.1.2 Plant Preparation

Lawsonia inermis L. (= L. *alba* Lamk.) (IPNI database, WFO (2020), Plant list accessed in September 2020), belonging to Lythraceae family, is a very widespread medicinal plant and natural dye in the world. It is collected in botanical garden Henri Gaussen (JBHG) of Toulouse during the seed-bearing stage in 2018. Voucher specimens were identified by Doctor Fatiha El Babili and deposited at JBHG herbarium. *Lawsonia inermis* also called "henna" grows in the JBHG ethnobotanical spiral. The aerial floral part is dried away from light in our airy dryer and then sprayed with an electric grinder. To make extracts to be tested, 1 g of drug is extracted with 100 ml water at 50°C during 30 min.

Argania spinosa (L.) Skeel Sapotaceae (IPNI database, WFO (2020) seeds were purchased in province of Essaouira and brought back to JBHG during spring 2019. They were also identified by Dr. Fatiha El Babili. For histochemical study, it is leaves that grow in one of our botanical garden greenhouses that will be used. A preliminary study on dry leaves reported from Morocco was carried out to verify exact identification and specially to verify if no significant difference exists between samples, since this tree is plant endemic to Morocco. Our specimen has bloomed, this summer but gives no seed for moment.

For antioxidant activity study by UV spectrophotometric test, it is seed testa that is chosen. Because in view of TLC, it clearly appeared that qualitatively seed testa was the richest in substances having an antioxidant activity. The seed part is dried away from light in our airy dryer and then sprayed with an electric grinder. To make extracts to be tested, 1 g of drug is extracted with 100 ml water at 100°C during 30 min. Working from traditional uses, we deliberately chose to work only on extracts that can be edible for medicinal use (with water and ethanol).

2.1.3 Methods for Assessing Antioxidants Presence in Plants

The method was developed by Blois (1958) to determine antioxidant activity, by using a stable free radical α,α-diphenyl-β-picrylhydrazyl (DPPH; $C_{18}H_{12}N_5O_6$, M = 394.33). The assay is based on antioxidants scavenging capacity measurement. The odd electron of nitrogen atom in DPPH is reduced by receiving hydrogen atom from antioxidants to corresponding hydrazine (Kedare & Singh, 2011).

The free radical test DPPH• (2,2-DiPhenyl-1-PicrylHydrazyl) is used. It is a stable free radical which acts by combining with other free radicals. This compound was one of the first free radicals used to study phenolic compound structure-activity relationship. This is a widely used test because it is simple and relatively reliable. DPPH is a black powder made up of stable free radical molecules. This is soluble in methanol or ethanol. This radical has a free electron on an atom of Nitrogen Bridge. The delocalization of this electron results in blue-violet coloration characteristic of reagent. When DPPH reacts with an antioxidant, a hydrogen atom is attached to radical. This fact results in loss of colour.

This allows to measure effectiveness of an antioxidant and that reaction can be followed in spectrophotometer between 515 and 518 nm.

DPPH solution preparation: DPPH solution preparation (Blois, 1958): 3.7 mg of DPPH powder are diluted in 25 ml of methanol or ethanol, leading to a concentration of 375 mM. The initial DPPH concentration should give absorbance values less than 1.0 for 50 to 100 µM (Blois, 1958) and between 1 and 1.2 for 375 mM. The powder must be prepared in advance and solution must be well stirred, since solubilisation is difficult. The solution can only be stored for a few days (1 to 3) in refrigerator and several weeks in freezer because DPPH stock solution slowly deteriorates.

2.1.4 Extracts Preparation

1g of dried leaf powder for *Lawsonia inermis* and 1 g of fruits and leaves powder for *Argania spinosa* are extracted in 100 ml of water heated to 100°C during 30 min (decoction). The same procedure was carried out for ethanolic extracts, only temperature is changed to 50 ° C. The solution is left alone for half an hour. The solution is then filtered using funnel and filter paper and then stored in freezer. 1 ml of aqueous or ethanolic extract is then diluted in 50 ml of water or ethanol and the mother solution S1 are thus obtained. The stock solution contains 10 mg of plant powder in 50 ml of water or ethanol. The concentration is therefore 0.2 mg/ml. When there is immediate discoloration of solution, an additional dilution game is applied under visual observation to properly regulate procedure for each plant extract type. Then, for each extract to be analysed, we carried out tests including 1 ml of water, 1 ml of test solution and then 1 ml of DPPH. This preparation is then put into obscurity for 40 minutes.

2.1.5 Absorbance Measurements

The absorbance is measured at reaction end; it is carried out at 517 nm. According to results, dilution range is adjusted to obtain a curve reflecting DPPH consumed percentage relative to concentration. Indeed, when solution reacts immediately, it is necessary to dilute solution because it means that all DPPH has reacted immediately. Conversely, when absorbance remains the same throughout reaction, it means that solution is too diluted. We carried out a kinetic curve to estimate solution reaction time to be analysed with DPPH. The leaves were picked and then dried for at least a week. They were then reduced to powder by means of mortar and pestle. The powder was then extracted with water or ethanol, depending on case studied. This extract is analysed by absorption spectrometer. It has been seen previously that, in antioxidant presence, DPPH radical is reduced: a solution discoloration is observed. The absorbance decay is measured by spectrophotometer and corresponds to decrease in DPPH radical concentration. The results of absorbance reduction measurements (IC_{50}) can be represented as a percentage:

% DPPH consumed = 1-(Absorbance in antioxidant presence/Absorbance without antioxidant)× 100

Extracts antioxidant activity was expressed as IC_{50}, defined as concentration of test material required to cause 50% decrease in initial DPPH concentration. Trolox was used as standard. All measurements were performed in quintuplicate or sextuplicate.

2.1.6 Statistical Analysis

All data were expressed as means ± standard deviations of triplicate measurements. The confidence limits were set at $P<0.05$. Standard deviations (SD) did not exceed 5% for majority of values obtained.

2.2 ICH Validation of Antioxidant Study

2.2.1 Standard Solution Preparation

A 150 mM stock Trolox solution was prepared in methanol by transferring required amount (1.88 mg) of Trolox into 50 mL of methanol. Then, a series of volumetric flasks of 10 mL with variable fractions were completed with methanol to prepare different standards in concentration in range 0.0024 and 0.024 mg/mL. All other solutions were stored in freezer in darkness when not in use.

2.2.2 Calibration Curves

To prepare standard solutions, accurately weighed amounts of Trolox standard (0.0024 and 0.024 mg/mL) were dissolved in methanol for analysis. The standard solutions were analysed and run for calibration curves. Calibration graphs were plotted subsequently for linear regression analysis of % consumed DPPH with amount of standard.

2.2.3 Sample Preparation

An aliquot of vegetable drugs powders (20-40 meshes, 1-10 g) was extracted, by simple ethanol extraction method or by decoction, 8 to 10 times its weight in appropriate solvent, as appropriate, for 30 minutes at 50°C for alcohols (ethanol or methanol) and at 100°C for the decoction with water. The resulting macerated and/or decocted material was filtered and then dried under reduced pressure (20 mm Hg) and a temperature below 50°C to produce an alcoholic and/or aqueous extract, depending on case. The appropriate amount of each extract was dissolved in 10 mL of methanol and or water, respectively. The solution was filtered by a 0.45 µm syringe filter before UV dosing. All separations were performed at room temperature.

2.2.4 Validation of DPPH Method

The Trolox analysis was validated considering linearity, LD, precision (repeatability, intermediate precision, and reproducibility), accuracy and robustness. The parameters validation will be carried out considering Q2 (R1) guidelines of International Conference on Harmonisation (Q2r1-Fra.Pdf; ANSES_Guide Validation.Pdf; Guide technique pour l'élaboration des monographies). For plant drugs analysed, same protocols will be followed. Here are parameters studied.

2.2.5 Linearity Study

Standard solutions containing Trolox were pre-dosed in ethanol from a freshly prepared solution (0.037mg /mL) to construct calibration curve. Regression analysis using the least squares method was performed for data obtained. The working solutions containing control and plant extract were prepared extemporanely in accordance with above requirements to plot calibration curve.

2.2.6 Limits of Detection (LD)

The standard solution containing all authentic compounds was diluted with methanol to provide appropriate concentrations. The limit of detection for each analyte was determined when ratio of testing peak signal to noise was greater than 5.

The developed method sensitivity was studied by calculating LD, the only one necessary for spectrophotometry. This was achieved by preparing a series of drug solution concentrations. The LD

was specified by an adequate dilution of known Trolox concentration until mean responses were 3- or 10-times standard deviation of responses for six measurements.

2.2.7 Precision Study

The analytical procedure precision expresses narrowness of measurement of dispersion between measurements series obtained from several samples, from homogeneous sample; under prescribed conditions.The method developed precision can be considered at 3 levels: its repeatability, its intermediate fidelity and reproducibility. The precision shall be evaluated by means of sample solution. It is generally expressed in terms of variance, standard deviation, or coefficient of variation of a series of measurement results. Intra- and inter-day tests were used to prove developed method precision. The later was conducted by analysing the 100 % concentration namely 0.037 mg/mL of Trolox, with standard solution curve.

2.2.7.1 Repeatability

It is the precision obtained under identical operating conditions and in a short interval of time. It must be assessed: or based on at least 9 determinations covering specified (measuring) interval (example: 3 concentrations with 3 replicates for each concentration); or based on at least 6 determinations at 100% of the test concentration.

Repeatability is measured using a standard range of 10 concentrations with six replicates in the same day. A solution containing Trolox at 0.037 mg/mL was prepared, analysing concentrations between 0.0024 and 0.024 mg/mL. The analyte amount was calculated from its corresponding calibration curve.

2.2.7.2 Intermediate Precision

The intermediate fidelity is performed by measurements on 2 different days, by two different analysts and on two different devices (UV-JBHG and UV-Déodat). The variability measurements were used to determine intermediate fidelity of the developed assay method. A concentration standard solution containing Trolox was prepared. It was done by analysing different concentrations levels namely: 0.0024 to 0.024 mg/mL of standard solutions. Quantity for the analyte was calculated from its corresponding calibration curve.

Each sample was analysed in sextuplicate within the same day to determine the intraday variability. The inter-day reproducibility was determined by analysing the sample on two separate days and on two different devices.

2.2.7.3 Reproducibility

In fact, reproducibility must be determined by analysing sample in 2 different laboratories. It is expression of inter-laboratory variability. It is to be considered in case of analytical procedure standardisation. This analysis is not necessary in our case since we only work on method validation and not its standardization.

2.2.8 Accuracy Studies

The accuracy of an analytical procedure expresses agreement narrowness between the value accepted as conventionally true, or as reference value, and the value found. It was determined by conducting recovery tests (Silva et al., 2017).

Determination of accuracy was done when acquiring fidelity, linearity, and specificity data. Accuracy should be evaluated on basis of at least 9 determinations, with at least 3 concentrations covering specified (measuring) interval (example: 3 concentrations with 3 replicates for each concentration) (Q2r1-Fra.Pdf).

The accuracy shall be indicated by recovery percentage in terms of difference between mean obtained and conventionally true value, with corresponding confidence intervals between 80 % and 120 %. Accuracy is evaluated from obtained results for analytical procedure linearity study, using pure Trolox as reference substance.

Then, to further evaluate developed assay recovery, amounts of Trolox calculated ($m_{calculated}$) were calculated from Trolox standard calibration curve, and amounts of Trolox measured ($m_{measured}$) were measured directly.

Each sample was analysed in five times. The analyte concentration was determined from the corresponding calibration curve, and recovery of analyte measurement was calculated by the following equation:

$$\text{Recovery (\%)} = (m_{measured} / m_{calculated}) \times 100$$

Where $m_{measured}$ is Trolox amount measured above and $m_{calculated}$ is the calculated amount of Trolox, calculated with calibration curve.

An appropriate amount of Trolox was weighed and then analysed. After that, each sample was analysed sextuplicate. In brief, different Trolox solution concentration levels using methanol as solvent were prepared. Three different Trolox concentrations solutions prepared using methanol (0.029, 0.037 and 0.044 mg/L) were used. Then concentrations (x) of resulting solutions were calculated using calibration curve.

2.2.9 Robustness

Robustness of developed method was measure of its ability not to be affected by weak, deliberate changes in factors associated with procedure; it gives an indication of procedure reliability under normal application conditions. The typical variations worked are following different devices (2 types of spectrophotometers in 2 different structures) and intervention of 2 different analysts. The stability of solutions subject to analysis (over a shelf life in freezer for 6 months) was not yet totally studied.

2.2.9.1 Statistical Analysis

All data were expressed as means ± standard deviations (SD) of triplicate measurements. The confidence limits were set at $P < 0.05$. SD did not exceed 5% for majority of values obtained.

3. Results and Discussion

The antioxidant activity evaluation by DPPH absorbance changes should be carefully interpreted since DPPH absorbance at 517 nm is decreased by light, oxygen, pH, and solvent type in addition to antioxidant[5]. The maximum uptake of Trolox control, in DPPH test, was detected at 517 nm and results are presented in Tables 1 to 5.

The determining antioxidant activity method, using DPPH radical, was simple, sensitive, precise, economical, and fast to be used in routine analysis; in our screening project, not only in extracts, as well as in raw materials study.

We have chosen to work on aqueous, alcoholic, and hydro-alcoholic extracts, because we seek through our screening work to identify plants traditionally used in Pharmacopoeias, still active. In Pharmacopoeias, only water and ethanol are used by populations since they are available and edible solvents. The other organic solvents remain reserved for research. Knowing that DPPH method can be used for aqueous and non-polar organic extracts, we can therefore consider study of both hydrophilic and lipophilic antioxidants (Prior, Wu, & Schaich, 2005). The method advantage of evaluating antioxidant activity is that DPPH radical, once reaction time, is sufficient to allow it to react slowly, may react even with low levels of antioxidants in plant extracts tested ("Antioxidant Activity-Medallion Labs,"n.d.,).

3.1 Analytical Method Validation Parameters

The developed method was validated in accordance with ICH guidelines (Q2) (R1) (Q2r1-Fra.Pdf).

3.1.1 Linearity

For Trolox:

The calibration curve consisted of ten different concentrations in range of 0.049 and 0.113 mg/ml for Trolox solution. The regression equation gives at the correlation coefficient superior to 0.99 between standard concentration (x) and mean absorbance (n = 3) shows a good linearity of standard curve. The calibration curve with regression equation was y = 2337.6 x + 6.8751 with a good correlation coefficient (0.9999). The curve was obtained by plotting mean DPPH (n = 5) consumed % (y) based on analyte (x) concentration in mg per mL (Figure 1).

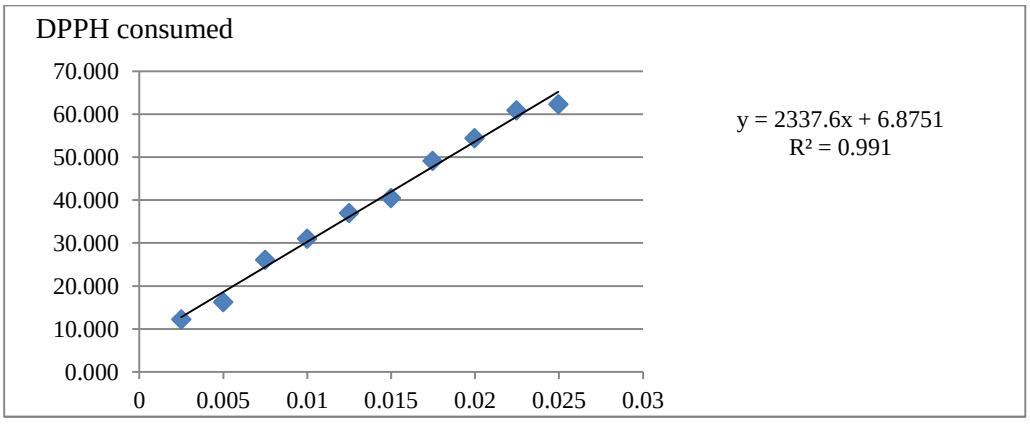

Figure 1: % DPPH consumed based on Trolox concentration

The spectrophotometer linearity was established throughout range measurement from concentration test dilutions. Each solution is measured by five or six repetitions. The UV assay linearity objective is its ability, in each interval, to provide results directly proportional to tracer studied concentration. Linearity shows that test results were directly proportional to amount extracted.

$$100\%f_{observed}=(p\text{-}2)\frac{r\text{^}2}{1-R\text{^}2}$$

$$F_{observed}=(10-2)\frac{0,991\text{^}2}{1-0,991\text{^}2}=438,45$$

$$F_{critical}=5,32 \text{ car } V1=1 \text{ et } V2=10-2=8$$

$$F_{observed}>F_{critical} \Rightarrow \ 438.45 > 5.32 \text{ so regression is significant.}$$

The calculated factor $F_{observed}$ is greater than tabulated $F_{critical}$. The slope being significant, there is linear dependence between Trolox concentration and % DPPH consumed, at threshold of probability tested (5%). The DPPH measured % linearly depends on Trolox tracer quantities, in the field tested.

For plants drug analysed:

The regression equation obtained for each extract with a correlation coefficient all superior to 0.99 between concentrations (x) and DPPH % shows a good linearity for all plants studied (Table 1).

Table 1: Results for Trolox and plants studied linearity study

Plant drug extracts studied.	Aqueous extract concentrations (mg/mL)	Regression equation - Standard curve	Correlation coefficient or r^2	F_{obs} (F_{crit} = 4.28)
Argania spinosa fruit pericarp	0.0025 à 0.015 mg/mL	Y = 4965.4 X + 11.315	0,9931	993 So validated linearity
Argania spinosa fruit almond	0.0045 à 0.015 mg/mL	Y = 5886.2 X - 16.85	0,9932	360.18 So validated linearity
Argania spinosa leaves	0.0075 à 0.025 mg/mL	Y = 2027.6 X – 6.1693	0,997	9.20 So validated linearity
Lawsonia inermis leaves	0.0025 à 0.025 mg/mL	Y = 1534.1 X – 3.256	0,991	8.20 So validated linearity
Trolox (standard)	0.049 and 0.113 mg/mL	Y = 2337.6 x + 6.8751	0.999	438.45 So validated linearity

r^2: Correlation coefficient of regression equation, limit of detection: S/N = 5

3.1.2 Precision

3.1.2.1 Repeatability

The relative standard deviation is variation coefficient absolute value. It is generally expressed as percentage. RSD is equal to standard deviation in relation to average and multiplied by 100.

Table 2: Results for Trolox and plants studied repeatability study

Studied samples	% RSD for Inter day tests	Average % of RSD
Argania spinosa fruit pericarp	0.069 to 0.478	0.237
Argania spinosa fruit almond	0.063 to 3.283	1.524
Argania spinosa leaves	0.087 to 0.478	2.178
Lawsonia inermis leaves	0.168 to 1.302	4.113
Trolox	0.069 to 0.478	0,057

The variation coefficient was taken as repeatability measures. Since variation coefficient is always less than 10%, determination method provides consistent results (Table 2). The method is precise.

3.1.2.2 Intermediate Fidelity: Dosages Carried Out from One Day to the Next

Table 3: Results for Trolox and plants studied intermediate fidelity study

Tested samples Intraday (n = 2)	DPPH consumed. average % in J0	DPPH consumed average % in J1	$F_{calculated}$	F-Test < ($F_{critical}$)
Argania spinosa fruit pericarp	94.10 ± 0.02	92.09 ± 0.01	3.71	< 5.05 - valid assumption
Argania spinosa fruit almond	86.79 ± 0.02	89.06 ± 0.01	1.58	< 5.05 - valid assumption
Argania spinosa leaves	45,56± 0.001	31,79 ± 0,32	0,0048	< 0,19 - valid assumption
Lawsonia inermis leaves	60.15± 0.131	60.31± 0,151	1.21	< 6.39 - valid assumption
Trolox	62.253± 0.001	62.391 ± 0,001	0,085	< 0,11 - valid assumption

The equal variances test (F-test) was taken as a measure of the intermediate precision.

Statistical comparisons of DPPH 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 tested plant extracts and Trolox standard. The means of DPPH consumed % are not significantly different (Table 3) because in F-test the $F_{calculated}$is always inferior to $F_{critical}$.

3.1.2.3 Reproducibility

The developed method reproducibility was achieved by analysing Trolox by different analysts using similar conditions.

Dosages carried out on two different spectrophotometers (inter-laboratory test): Equipment (n = 2): S-JBHG and S-Déodat

Specifically, intraday precision (*repeatability*) can be defined as analytical procedure use within a laboratory under a short period time through analysing six replicates on the same day by the same analyst using the same equipment. On the other hand, inter-day precision (intermediate precision) implies variations evaluation in analysis when method is used within laboratory on different days and by different analysts (Q2r1-Fra.Pdf).

The % RSD for tests with different devices (S-JBHG and S-Déodat) values for intra-assay precision and intermediate precision for concentration level of 0,0249 were below 2 % (Table 4) indicating developed method good reproducibility.

Table 4: Results for Trolox and plants studied intermediate fidelity study

Tested samples	DPPH consumed average % in S-JBHG	DPPH consumed average % in S-Déodat	% RSD (variation Coefficient) for tests with different devices
Argania spinosa fruit pericarp	94.123 ± 0.164	92.717 ± 0.139	0.81
Argania spinosa fruit almond	89.035 ± 0.112	90.787 ± 0.088	1.032
Argania spinosa leaves	44.496± 0.406	45.985 ± 0.126	1.844
Lawsonia inermis leaves	61.122 ± 0.158	60.783 ± 0.095	0.378
Trolox	60.742 ± 0.062	62.270 ± 0.035	1.311

Conclusion: There are no significant differences in UV antioxidant assays between the two devices.

3.1.3 Accuracy

Trolox accuracy is obtained by recoveries between 95.48 % and 102.90 % (Table 5). The results obtained support method developed precision. The accuracy was reported as % recovery ± standard deviation. Accuracy values obtained were in the range of 95 to 110 % as indicated in Table 5.

Table 5: Accuracy results for Trolox standard and plants studied

Plant drug extracts studied	Test concentrations range - mg/L	Theoretical value in % of test sample	% DPPH consumed *measured*	% DPPH consumed *recalculated*	Recovery rate range
Argania spinosa fruit pericarp	0.005 to 0.015	100	69.17±0.13	79.47	99.36 to 107.06
Argania spinosa fruit almond	0.004 to 0.015	100	56.12±0.50	63.94	95.61 to 108.08
Argania spinosa leaves	0.007 to 0.025	100	20.78±0.25	22.21	95.87 to 107.23
Lawsonia inermis leaves	0.015 to 0.025	100	17.80±0.12	17.80	95.37 to 103.29
					% Recovery* (Mean ± SD)
Trolox	0.029	80	64.74	63.84	101.41 ± 0.110
	0.037	100	62.27	65.21	95.48 ± 0.054
	0.044	120	79.65	77.41	102.90 ± 0.112

*Indicates mean of five determinations (n = 5); SD: Standard deviation.

The good accuracy results obtained reveal developed method potential for analyte quantification in herbal extracts. The results show that recoveries obtained from the 100 % standard are homogeneous and are all between 95% and 110% (Table 5). The method is therefore accurate in field tested: at 0.029, 0.037 and 0.044 mg/L for Trolox and with range 0.004 to 0.025 for herbal extracts. The obtained results support developed method accuracy.

3.1.4 Specificity

The developed method was found selective and specific as there is no interferences occurred as reflected by accuracy results.

3.1.5 Robustness

Table 6: Robustness results (different equipment) for Trolox standard and plants studied

Plant drug extracts studied	Average consumed DPPH % - S-JBHG	Average consumed DPPH % - S-DEODAT	F-test $F_{calulated}$ and $F_{critical}$
Argania spinosa fruit pericarp	94.102 ± 0.023	92.68 ± 0.021	1.09 < 5.05
Argania spinosa fruit almond	86.79 ± 0.020	90.79 ± 0.006	3.09 < 5.05
Argania spinosa leaves	45.56 ± 0.001	46.02 ±0.020	0.07 < 0.19
Lawsonia inermis leaves	62.71± 0,01	53.62 ± 0.01	1.61 < 5.05
Trolox	62,27 ± 0035	60.74 ± 0.062	3.26 <9.27

Table 7: Robustness results (different analyst) for Trolox standard and plants studied

Plant drug extracts studied	Average consumed DPPH % - analyst 1	Average consumed DPPH % - analyst 2	F-test $F_{calulated}$ and $F_{critical}$
Arganiaspinosa fruit pericarp	21.39 ± 0.01	35.69 ± 0.005	1.69 < 5.19
Arganiaspinosa fruit almond	26.05 ± 0,56	23.60 ± 0.12	4.62 < 5.05
Arganiaspinosa leaves	Nd (not determined)	nd	nd
Lawsoniainermis leaves	60.12 ± 0,01	59.13 ± 0.004	3.12 < 6.38
Trolox	62.50 ± 0.08	59.10 ± 0.04	1.86 < 6.38

The variations in stability subject to analysis, different equipment use, and 2 different analysts intervention gave good results (Tables 6 and 7), indicating current method robustness.

3.1.6 Application of UV Spectrophotometric Method in Screening

The method developed was successfully applied to determine the antioxidant properties of pure chemicals such as Trolox and plant extracts. In accordance with the ICH guidelines, assay values for all types of samples studied, i.e., plant raw materials (herbal decocted extracts) and pure substances were found to meet the standards. The results indicate a good adequacy of the DPPH assay method for the assessment of antioxidant activity in various plant extracts of the medicinal plants studied. This method will therefore be used routinely to carry out a phytochemical screening that will allow us to find the plants with the most promising properties among the 4000 of our heritage collections.

The linearity, accuracy and recovery rate of spectrophotometric method used to evaluate plant extract antioxidant activity are validated. The % of DPPH consumed changes linearly according to antioxidant amounts present in plant extract studied, when tested between 0.0024 and 0.0249 mg/L for each extract.

Under optimal conditions used in this study, calibration curve showed good linear regression, as shown in Table 1.The results in Table 4 demonstrate that analytical method developed is reproducible with good accuracy and sensitivity for antioxidant examined. Overall intra-day and inter-day variations are less than 10%. The recovery tests of DPPH consumed % give results given in Table 4, from which it is quite clear that recovery rates were in range of 95 to 110%.

4. Conclusion

Our botanical study provides a standard diagnostic tool to help for phytochemical properties preliminary identification before plant medicinal use. Moreover, our work relates on development of

a simple, sensitive, and specific UV method to quantify bioactive ingredient, in herbal drugs. The results demonstrate that developed method is accurate and reproducible and could be readily used as a suitable quality control method. A simple, reliable, accurate and reproducible spectrophotometric DPPH method for antioxidant properties determination in raw materials was successfully developed as per the ICH guidelines. The good analytical performance with regards to validation parameters was achieved. All validated data attained agree with ICH guidelines.

Good recoveries of Trolox were obtained in the range of 95.48 to 102.90 % (Table 5) in different samples confirming developed method accuracy. The developed method is thus recommended to be implemented as a qualitative control protocol in phytochemical screening, often necessary before starting a plants study with anticancer properties, among others.

Author Declaration Template

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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