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Research Article

Evaluation of Functional Stability and Batch-to-Batch Reproducibility of a *Castanea sativa* Leaf Extract with Antioxidant Activity

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Abstract. A growing body of evidence suggests that free radicals are generated by UV irradiation being responsible for skin injury. In this regard, the topical use of formulations composed of plant extracts with antioxidant activity could represent a useful strategy for the prevention of photoaging and oxidative-stress-mediated diseases. The aim of this study was to assess the reproducibility of the extraction method and the functional stability of a *Castanea sativa* leaf extract in view of its application as topical antioxidant. Measurements of 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity, total phenols (measured by the Folin Ciocalteu assay) and phenolic composition (high-performance liquid chromatography unit coupled to a UV detector) were carried out on three different batches. The influence of pH and temperature on the extract's DPPH scavenging activity was assessed in aqueous and glyceric solutions (0.025% *w*/*v*) over a 3-month period. Minor differences were found between the three extract batches for all the evaluated parameters, and therefore the reproducibility of the extraction method can be inferred. pH presented a great influence in the extract functional stability. Major antioxidant activity decrease was found at pH 7.1, while lower changes were observed at pH 5. Glyceric solutions were stable throughout the test period. At 40°C and pH 5, a marked decrease of activity was observed. Again, glyceric solutions were the most stable, even at 40°C. Proper selection of pH and solvent is mandatory to ensure the stability of the studied extract after being incorporated in semisolid forms. In view of these results, glycerine is proposed as the best vehicle for topical formulations incorporating *C. sativa* leaf extract, which should have a pH around 5.

KEY WORDS: batch reproducibility; *Castanea sativa*; DPPH; functional stability; total phenols.

INTRODUCTION

Photoaging is largely mediated by the overproduction of free radicals (1,2) and by impairment of antioxidant systems (3). Furthermore, UV-induced oxidative stress is thought to contribute to dermatoses such as psoriasis and skin cancer (4–6). In this regard, topical application of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system. This might be a successful strategy to diminish UV radiation-mediated oxidative damage in skin and/or to prevent oxidative-stress-mediated diseases (7–9).

Among the antioxidants, plant polyphenols have gained special interest over the past few years (10,11), especially taking into account that some of the synthetic antioxidants available raise safety concerns (12,13). *Castanea sativa* (Fagaceae) is a species of chestnut native in southeastern Europe and Asia Minor. Chestnut leaves are used in traditional medicine in the treatment of several diseases, such as bronchitis and cough (14). 1,1-Diphenyl-2-picryl hydrazyl (DPPH) scavenging activity, scavenging activity against superoxide anion and hydroxyl radicals, was previously reported for *C. sativa* leaves (15). Such antioxidant activity was suggested to be related with the phenolic composition (15). Rutin, hesperidin, quercetrin, apigenin, morin, galangin, kaempferol (16), and isoquercitrin (15) have been identified in *C. sativa* leaves.

Medicinal or cosmetic use of plant extracts must rely on appropriate quality, safety, and efficacy requisites. In this regard, batch-to-batch reproducibility is essential to ensure consistent quality. In this work, three independent batches were compared with respect to the phenolic composition and the antioxidant activity.

Besides reproducibility of the extraction procedure, stability is also important for the quality of herbal extracts. Data of the extract's stability is of utmost importance in the guidance of the formulation step, with respect to the selection of solvent and other excipients and the formulation pH, among others. Due to the inherent complexity of herbal products, there is no single stability-indicating assay or parameter that fully describes their stability characteristics. For instance, the quantification of only a few compounds does not give a comprehensive and accurate assessment of all active constituents. Moreover, knowing precisely which compounds are responsible for the therapeutic activity is also a difficult task because these compounds often work synergistically.

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Table I. DPPH Scavenging Activity, Total Phenols and Extract Yield for the Three Batches

	Batch				
	Ascorbic acid	1	2	3	Mean
DPPH IC_{50} (μg/mL) $n=3$	4.93±0.36[b]	12.13±0.21[b]	11.67±0.32[b]	12.95±0.63[b]	12.32±0.37[b] RSD(%)=5.2
Total phenols (mg GAE[a]/g) $n=3$		263.1±1.6[c]	272.7±3.7[c]	273.9±3.2[c]	269.9±5.95[c] RSD(%)=2.2
Yield (mg)		1180	1168	1212	1186.7±22.7[c] RSD(%)=1.9

[a] Gallic acid equivalents
[b] Mean ± SEM
[c] Mean ± SD

In this work, the extract's stability was evaluated using the DPPH assay, originally developed by Blois (17), which is an easy, inexpensive, fast, and precise methodology to measure the antioxidant activity. DPPH is a stable free radical that can accept an electron or hydrogen radical converting it into a stable, diamagnetic molecule. It presents strong absorption of visible radiation at 517 nm, and when its odd electron becomes paired off the absorption decreases stoichiometrically with respect to the number of accepted electrons. Such methodology has been used to evaluate the functional stability of antioxidants either alone or incorporated in dosage forms (8,18). It is noteworthy that this method measures the antioxidant activity of the whole extract taking therefore into account possible synergisms. Furthermore, there is no need for markers which could be troublesome when dealing with complex matrixes like plant extracts.

In order to gain useful information to guide the formulation step, the extract functional stability in aqueous solutions at pH 5 and 7.1 (which are relevant for topical application) and in 85% glycerine was evaluated. The effect of temperature (4°C, 20°C, and 40°C) in the functional stability was also evaluated for those solutions where the extract presented better stability.

MATERIALS AND METHODS

Chemicals

The standards, Folin Ciocalteu reagent, and 1,1-diphenyl-2-picryl hydrazyl were purchased from Sigma (St. Louis, MO, USA) and Extrasynthése (Genay, France). Methanol, ascorbic acid, sodium carbonate, and formic acid were obtained from Merck (Darmstadt, Germany). Glycerine 85% (*w*/*w*) was obtained from Fluka Chemie (Steinheim, Germany). Ethanol 96% was purchased from Aga (Lisbon, Portugal). 2-Amino-2-hydroxymethyl-1,3-propanediol (Tris) and sodium acetate were purchased from Vaz Pereira (Lisbon, Portugal).

Plant Material

C. sativa leaves were collected during July 2003 in Mirandela (coordinates 41°29′ N 7°11′ W), Northern Portugal, and dried at room temperature for 3 weeks. A voucher specimen is preserved in our laboratory for further reference.

Preparation of the Extracts

The dried leaves (4 g) were grounded and sieved (500 μm) and extracted five times (20 min, 100 mL, 500 rpm) with ethanol/water (7:3) at 40°C, and then the solutions were filtered with a glass filter funnel (G4). The extracts were gathered, the ethanol was evaporated at 40°C under vacuum, and then the resulting aqueous mixtures were lyophilized (FreeZone 4.5 L, Labconco Corporation, Kansas City, USA).

Determination of Total Phenolics

The amount of total phenolics in the extract was determined using the Folin Ciocalteu colorimetric method, according to a described procedure (19). Briefly, 1 mL of Folin Ciocalteu reagent was added to 300 μL of extract dissolved in ethanol/water (7:3) (500 μg/mL), followed by the addition of 5 mL of 20% sodium carbonate solution. The mixture was made up to 10 mL with deionized water and thorough shaking. The absorbance was read at 735 nm in a UV–VIS Spectrophotometer, model V 530 (Jasco Corporation, Tokyo, Japan), after 20 min of incubation at room temperature.

The phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry extract. The measurements were performed in triplicate.

Quantification of Phenolic Compounds

The lyophilized extract was dissolved in ethanol/water (7:3) and filtered (0.22 μm) before being analyzed. The

Table II. Validation Parameters of the HPLC Method for the Analysis of *C. sativa* Leaf Extract

Phenolic compound	Intra-day precision (RSD) (%)	Inter-day precision (RSD) (%)	Linearity	Linear range (mg/mL)
Rutin	1.8	5.3	$y=1.74\times10^7x-7{,}713$; $R^2=1.0000$	0.0039–0.5
Chlorogenic acid	1.7	5.6	$y=2.71\times10^7x-28{,}888$; $R^2=0.9999$	0.0039–0.5
Hyperoside	0.5	2.8	$y=2.11\times10^7$x$-51{,}357$ $R^2=0.9995$	0.0039–0.5
Ellagic acid	2.0	5.6	$y=1.49\times10^7$x$+458$; $R^2=0.9987$	0.000977–0.125

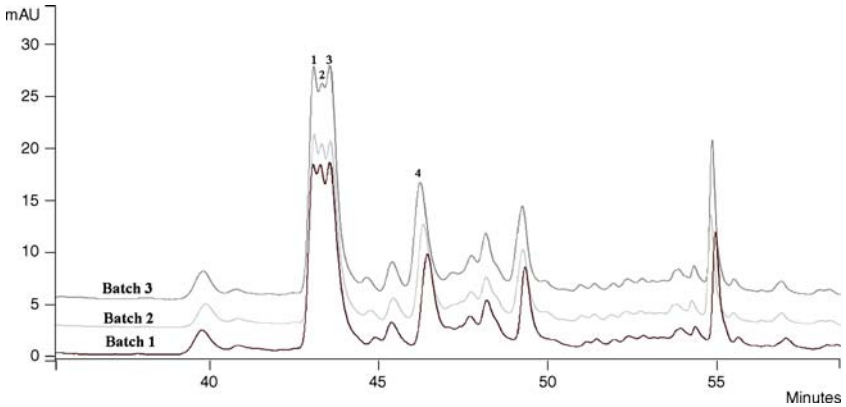

Fig. 1. HPLC chromatograms obtained from three batches of *C. sativa* leaf extracts, λ= 350 nm. *1* Hyperoside, *2* isoquercitrin, *3* rutin, *4* ellagic acid. Chlorogenic acid is omitted from this graph because it is quantified at a different wavelength

extract (20 μL) was analyzed on a high-performance liquid chromatography (HPLC) unit coupled to a UV detector both from Varian (Palo Alto, USA) using a reversed-phase Spherisorb ODS2 column (250×4.6 mm, 5 μm particle size) from Merck (Darmstadt, Germany) with a C18 guard column. The solvent system was a gradient of water/formic acid (19:1) (mobile phase A) and methanol (mobile phase B), starting with 5% methanol and progressing to obtain 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 45% B at 39 min, 45% B at 42 min, 50% B at 44 min, 55% B at 47 min, 70% B at 50 min, 75% B at 56 min, and 80% B at 60 min at a solvent flow rate of 0.9 mL/min (20). Each batch was analyzed in triplicate. The phenolic compounds were previously identified by their UV spectra and their HPLC retention time, using co-injection with the authentic standards for identity confirmation. Phenolic compound quantification was carried out using linear calibration graphs obtained from the correspondent standard solutions. The quantification was conducted at 320 nm for chlorogenic acid and at 350 nm for the other polyphenols. Data were processed on the Star Chromatography workstation software, version 6.3 (Varian, Palo Alto, USA).

Validation of the HPLC Method

Three independent stock solutions containing the reference compounds were prepared in methanol and diluted to a series of five concentrations which established the calibration curves. Intra-day precision was evaluated by the injection of a standard solution using six replicates, in the same day. For inter-day precision, measurements of the same standard solution on three different days were conducted. The precision was expressed as relative standard deviation (RSD).

DPPH Scavenging Assay

The scavenging capacity of the stable 1,1-diphenyl-2-picryl hydrazyl free radical was measured by monitoring its reduction, reflected in the absorbance decrease at 515 nm, according to a described procedure (21) with modifications. Reaction mixtures contained DPPH (190 μM) and the extract at different concentrations (100, 50, 25, 12.5, 6.25, 3.12, and 1.56 μg/mL) dissolved in ethanol/water (7:3) in a final volume of 200 μL. After 20 min of incubation, the absorbance was measured at 515 nm in a microplate reader (ELX 808 IU, Bio-Tek Instruments, Vermont, USA). The effects were expressed as the percentage DPPH reduction, and the concentration that was able to scavenge 50% of the radical (IC_{50}) was calculated from linear graphs. Ascorbic acid was used as positive control. Three independent experiments were performed in duplicate.

Evaluation of Functional Stability

The lyophilized extract was dissolved (0.025% *w*/*v*) in Tris buffer (pH 7.1, 50 mM), sodium acetate buffer (pH 5,

Table III. Phenolic Composition of the Three Batches

Phenolic compound (mg/g)	Batch 1	Batch 2	Batch 3	Mean ± SD
Chlorogenic acid	2.23±0.06	2.21±0.07	2.25±0.06	2.23±0.02 RSD (%)= 1.1
Hyperoside	3.73±0.17	3.72±0.01	4.24±0.05	3.90±0.30 RSD (%)=7.6
Isoquercitrin[a]	3.55±0.15	3.36±0.03	3.18±0.08	3.37±0.18 RSD (%)=5.5
Rutin	6.76±0.17	5.77±0.27	6.06±0.09	6.20±0.51 RSD (%)=8.2
Ellagic acid	3.00±0.07	3.01±0.09	3.20±0.04	3.07±0.11 RSD (%)=3.6

n=3
[a] Quantified as hyperoside

50 mM), and glycerine 85% (*w*/*w*) and stored for 3 months at 20°C. The tests were also conducted at 4°C and 40°C with the solvents that presented the best stability. Scavenging effect against DPPH was evaluated at the beginning of the assay and after 1, 7, 30, and 90 days, as previously described with the following modifications: reaction mixtures contained DPPH (150 μM) and the extract at different concentrations dissolved in ethanol 96% in a final volume of 200 μL. pH measurements (pH meter, Mettler Toledo, Columbus, USA) were taken throughout the test period to exclude the potential interference of pH modifications.

Statistical Analysis

Mean and standard deviations (SD) or standard error of the mean (SEM) were computed with SPSS 15.0 for Windows software (SPSS Inc., Chicago, USA). The linear regression analysis was made with MS Excel 2003 (Microsoft, Redmond, USA).

RESULTS

The results obtained for the total content of phenolics, DPPH scavenging activity, and extract yield of the three batches are shown in Table I. Minor differences between batches (RSD less than 5.2%) were observed for all parameters.

The validation parameters of the HPLC method are presented in Table II. HPLC chromatograms were similar among the three batches (Fig. 1). The amounts of phenolic compounds (Table III) found for each batch presented some variation (RSD lower than 8.2%), which can be attributed to the complexity of the plant extract and the variability associated with the methodology. Furthermore, the flavonoids with higher variability (rutin, isoquercitrin, and hyperoside) co-elute slightly, which makes their quantification more difficult. Taken together, these results demonstrate the reproducibility of the extraction procedure.

pH presented a great influence in the extract functional stability, evaluated with DPPH assay. Results are expressed as the concentration that was able to scavenge 50% of the radical (IC_{50}), and thus the higher the value of IC_{50} the lower is the scavenging activity. Major activity decrease was found at pH 7.1, while lower changes were observed at pH 5. The modifications at pH 7.1 were detected early on day 7 and continued to progress with time. Glyceric solutions were the most stable throughout the test period (Fig. 2). Minor pH variations on the different solutions during the time of this study were observed (data not shown), and thus the lack of stability observed under some storage conditions cannot be attributed to pH modifications.

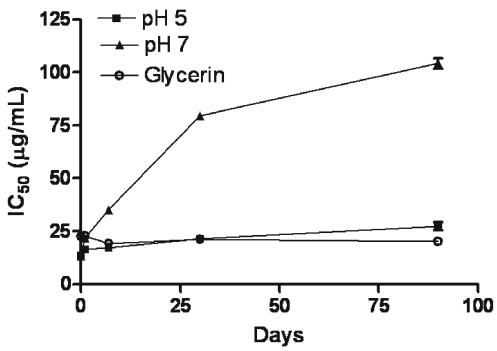

Fig. 2. Functional stability of *C. sativa* leaf extract in different solvents: aqueous buffered solution at pH 5 and 7.1 and glycerine 85%. T=20°C. Mean ± SEM, n=3

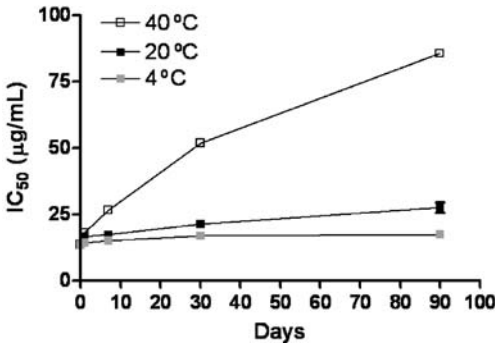

Fig. 3. Functional stability of *C. sativa* leaf extract at pH 5 during storage for 3 months at different temperatures. Mean ± SEM, n=3

Temperature also influenced the functional stability of the *C. sativa* leaf extract. At 40°C and pH 5, a marked decrease of activity was observed on day 7 followed by further decreases over time (Fig. 3). Again, glyceric solutions were the most stable, even at 40°C (Fig. 4). The storage temperature had little effect on the functional stability of *C. sativa* extract dissolved in glycerine 85%.

Storage at 4°C, pH 5 seems to protect the extract from degradation. The IC_{50} values remained practically unchanged during 90 days (Fig. 3).

DISCUSSION

Batch variability is a common drawback in the manufacture of plant extracts. The extraction method used in this work was previously optimized with respect to solvent, duration and number of extraction steps, and temperature (22). The overall results showed that the extraction method is reproducible, yielding extracts with suitable quality in terms of the content of phenolic compounds and antioxidant activity. This is a primary requisite for the potential application of this extract in cosmetic/pharmaceutical formulations.

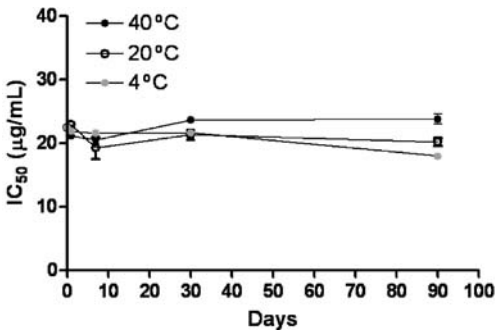

Fig. 4. Functional stability of *C. sativa* leaf extract in glycerine 85% (*w*/*w*) during storage for 3 months at different temperatures. Mean ± SEM, n=3

The phenolic composition of the *C. sativa* extract comprises chlorogenic acid, hyperoside, ellagic acid, rutin, and isoquercitrin. The major compound was found to be rutin, as shown in Table III. DPPH scavenging activity has been reported for the above-mentioned polyphenols (21,23,24); thus, their contribution to the extract free-radical scavenging activity can be postulated.

At pH 7.1, a marked decrease of activity was observed. Taking into account that polyphenols are responsible, at least partially, for the antioxidant activity, we can hypothesize that the loss of activity might be due to the degradation of the phenolic compounds contained in the *C. sativa* extract. The influence of pH on the stability of polyphenols was previously reported and claimed to be strongly dependent on the structure of the phenolic compound. Chlorogenic acid was found to be sensitive to high pH conditions, while rutin resisted major pH-induced degradation (25). Buchner *et al*. found that the amount of rutin remained almost unchanged at pH 5, while it depleted around 20% (in 300 min) when pH was adjusted to 8 (26). This is in accordance with the results of this study, and it is of particular relevance because rutin is the major phenolic compound present in the extract. Rutin was reported to be more stable than its aglycone quercetin, which was correlated with the blockage of 3-hydroxyl group in the C-ring by a sugar moiety (26).

At pH 5 and 20°C, the extract was quite stable, but when stored at 40°C, it rapidly (from day 7) became less active. A negative effect of heat on the stability of some polyphenolic antioxidants has been previously described. Hyperoside and rutin were found to be less stable to heating than chlorogenic acid (27).

Glycerine is a polyalcohol used in topical formulations and cosmetics primarily for its humectant and emollient properties (28). The beneficial role of glycerine in the stability of polyphenols was previously demonstrated in a study were epigallocatechin remained stable at 50°C in a glycerine-based Carbopol gel. This fact was justified by the anhydrous character of the formulation, which resulted in the protection of oxygen/water sensitive substances (29). The glyceric solution used in this work was not completely anhydrous since it contained 15% of water, and therefore total protection from hydrolysis is not expected. Furthermore, hydrolysis of the quercetin glycosides (rutin, hyperoside, and isoquercitrin) would probably result in an increase in activity since their aglycone quercetin has been found to be more active than the respective glycoside forms (23,30). The role of glycerine in the stability of polyphenols is not fully clarified. Glycerine has three hydroxyl groups through which hydrogen bonds can be formed with water molecules, and as a consequence it can render water less available to participate in degradation reactions. The effect of glycerine in skin hydration is well documented (31). Glycerine-containing formulations produce long-lasting moisturization by binding and holding water and, at higher concentration, by minimizing water loss (32). Besides the role of glycerine on the protection of polyphenols, a positive effect of topical formulations with glycerine on skin hydration can be foreseen.

The DPPH scavenging assay used proved to be a suitable methodology for the evaluation of the functional stability of the studied plant extract because it allowed discrimination among the different storage conditions. This methodology is widely used to evaluate radical scavenging activity, even though this long-lived nitrogen radical is not biologically relevant. Comprehensive characterization of the antioxidant activity of the ethanol/water (7:3) *C. sativa* leaf extract studied in this work was previously carried out (33). Scavenging activity against several reactive oxygen species and reactive nitrogen species that are thought to be generated in human skin after UV irradiation was demonstrated, which reinforces the putative interest of the studied extract as topical antioxidant. The *C. sativa* leaf extract studied presented a tenfold higher antioxidant activity (evaluated with DPPH assay) and higher polyphenol content than reported by Barreira *et al*. for a leaf extract obtained with boiling water (34). The higher temperature used in the mentioned study might be the reason for the discrepancy especially considering the influence of temperature in the extract's antioxidant activity as demonstrated in the current work. Furthermore, it was previously established that hydroalcoholic mixtures are better extractive solvents than water for this particular plant (22).

CONCLUSION

Plant extracts with antioxidant activity are expected to provide protection against oxidative-stress injury after topical application. However, before formulating the topical forms, it is necessary to establish the stability of the plant extract. Our study demonstrated that pH and solvent selection are critical to ensure functional stability that will ultimately determine *in vivo* effectiveness. For the particular case of *C. sativa* leaf extract, formulations containing glycerine and pH close to 5 are suggested.

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