

Phcog Rev. : Review Article

Spectrophotometric Methods for Determination of Plant Polyphenols Content and their Antioxidant Activity Assessment: an Overview

Hicham Harnafi* and Souliman Amrani

Laboratory of Biochemistry, Department of Biology, Faculty of Sciences, 60 000 Oujda, Morocco.

*Corresponding author: E-mail: hicham.harnafi@etu.univ-lille2.fr.

ABSTRACT

Medicinal plants, as source of remedies, are widely used as alternative therapeutic tool for the prevention or treatment of many diseases. On the other hand, despite the remarkable progress in synthetic organic chemistry, over 25% of prescribed medicines in industrialized countries derived directly or indirectly from medicinal plants. Polyphenols, are the major plant secondary metabolites and represent the most studied phytochemicals. This family includes three major classes different by their specific chemical structure, we note: tannins, flavonoids, and anthocyanins. Many epidemiological and experimental studies have shown that flavonoid intake is inversely correlated with cancer, cardiovascular and cerebrovascular disease events. In this field, a number of polyphenols from medicinal plants have been shown to protect body against effects of oxidative stress. These phytochemicals react as free radical scavenging, reducing, and metal chelating substances. In this paper we present the major colorimetric techniques used to determine polyphenol content in medicinal plant extracts and the spectrophotometric assays employed to measure their antioxidant power.

KEY WORDS: Polyphenols, Dosage, Antioxidant Effect, Methods.

INTRODUCTION

Oxidative stress is involved in the pathogenesis of various chronic diseases, such as cardiovascular disease and cancer (1, 2). Antioxidants protect against free radicals and they are therefore essential in obtaining and preserving good health. Much attention has been given to polyphenols with strong antioxidant activities, which are ubiquitously present in a broad range of medicinal plants and dietary products. Furthermore, as reported by many investigators, polyphenol from medicinal and aromatic plant possess a high anti-oxidant potential due to their hydroxyl groups and protect more efficiently against free radical-related diseases such as atherosclerosis (3, 4, 5).

Polyphenols have powerful antioxidant activities. These phytochemicals are able to scavenge a wide range of reactive species, including hydroxyl radicals, peroxy radicals hypochlorous acid and superoxide radical. Phenolic substances can also inhibit biomolecular damage by peroxynitrite in vitro (6-8), although they are less good at doing this in the presence of physiological levels of HCO₃/CO₂ (8, 9). We note that peroxynitrite reacts with CO₂/HCO₃ to form reactive substances which are quickly scavenged by phenolics. Many polyphenols present a high potential to chelate transition metal ions such as copper and iron responsible for reactive species formation (10, 11). The antioxidant capacity of polyphenols is generally suggested to be responsible for their protection against cancer, neurodegenerative and cardiovascular diseases (12, 2, 13-15).

In this review we gave interest on the spectrophotometric methods used to quantify polyphenol in medicinal plant extracts and to assess their antioxidant activity.

Methods of Polyphenols Quantification in Plant Extracts

Dosage of total phenol

Total phenol content in plant extracts was generally

determined according to the Folin-ciocalteu method (16). This colorimetric method is based on the reduction of a phosphotungstate-phosphomolybdate complex by phenolics to blue reaction products in alkaline conditions. To 0.5 ml of plant samples adequately diluted were added 250 µl of Folin-ciocalteu reagent and 1.25 ml of 20 % aqueous sodium carbonate solution. After incubation 45 min in dark, the absorbance was spectrophotometrically recorded at 725 nm versus the blank (0.5 ml of plant sample was replaced by 0.5 ml of extracting solvent). The amount of total polyphenols in plant extracts was calculated from the calibration curve prepared, in the same conditions described above, with known polyphenols standard such as caffeic acid, gallic acid or catechin and expressed as mg standard equivalent/g of dry plant extract.

Quantification of flavonoids

The total flavonoid concentration was measured using a colorimetric assay developed by Jay et al. (17). The method is based on the quantification of yellow color produced by the interaction of flavonoids with AlCl₃ reagent. Briefly, To each 5 ml of analyzed solution of plant extract appropriately diluted, 2.5 ml of AlCl₃ reagent were added (133 mg crystalline aluminium chloride and 400 mg crystalline sodium acetate were dissolved in 100 ml of extracting solvent) and absorbances were recorded at 430 nm against a blank (5 ml of analyzed solution plus 2.5 ml of extracting solvent). The flavonoids content was determined from the calibration curve of standard flavonoids such rutin, quercetin or luteolin and expressed as mg standard equivalent/g of dry plant extract. Flavonoids content was also determined by the method proposed by Ough and Amerine (18) using formaldehyde for their precipitation. The flavonoids concentration was calculated as the differences between total phenols and non-flavonoids remaining in the supernatant.

Dosage of tannins

Total tannins content was determined by the Folin-Ciocalteu procedure, as described above, after their adsorption onto hide powder (collagen fiber powder prepared from bovine skin) (19), polyvinyl polypyrrolidone (PVPP), bovine serum albumin BSA, gelatin or casein (20). In brief, samples of plant extract were homogenized with adsorbent material and the mixture was stirred for 30-60 min; the preparation obtained was stored for 1-2 h at +4 °C to develop tannin-protein or tannin-PVPP complex. Then the pH was adjusted to pHi of protein used or acidified (pH 3) in case of using PVPP. After centrifugation at 4000 rpm/ 15 min, no adsorbed phenolics in the supernatant were determined by the Folin-Ciocalteu procedure as described above. Calculated values were subtracted from total polyphenol contents and the amount of total tannins expressed as mg standard polyphenol/g dry plant extract.

Methods for Assessment of Polyphenols Antioxidant Activity Radical scavenging activities

DPPH assay

The hydrogen atoms or electrons donation ability of polyphenol-rich extract was measured from the bleaching of purple colored methanol or ethanol solution of DPPH. This spectrophotometric method uses stable radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH) as a reagent (21, 22). Five ml of the polyphenol extracts dissolved in methanol or ethanol were added to 2.5 mL of a 0.1 mM solution of DPPH. After a 30 min incubation period at room temperature the absorbance was recorded against a blank at 517 nm. Scavenging of free radical DPPH was calculated according to the following formula: % inhibition = $(A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$; where A_{blank} is the absorbance of the control reaction (containing DPPH solution adequately diluted with methanol or ethanol) and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration.

Superoxide anion scavenging activity

The superoxide anion O_2^- was generated photochemically or by phenazine-methosulfate (PMS)-NADH method (23, 24). The inhibition of nitroblue tetrazolium (NBT)-reduction was used to determine the superoxide anion scavenging activity of the test samples. The reaction mixture was prepared by mixing 56 mM (NBT), 0.01 mM methionine, 20 mM NaCN and 0.05 M phosphate buffer, pH 7.8 or 12 mM PMS, 100 mM NADH, and 100 mM NBT in 0.1 M phosphate buffer ($K_2HPO_4-H_2PO_4$) at pH 7.8. Superoxide was measured spectrophotometrically by the increasing amount of the absorbance at 560 nm at 30 °C after 6 min incubation with samples at different concentrations. Concentration required to inhibit NBT reduction by 50% (IC_{50}) was calculated from dose-inhibition curves.

Peroxonitrite scavenging activity

Potassium oxoperoxonitrite (ONOOK) was produced from the reaction of solid KO_2 with NO-gas as described by Koppenol et al. (25). Peroxonitrite scavenging related to polyphenols or polyphenol-rich extracts was measured by the oxidation of dihydrorhodamine (DHR), as described by Kooy et al. (26). Fluorescence measurements were performed on a spectrophotometer at 37 °C with excitation and emission

wavelengths of, respectively, 500 and 536 nm. The effects are expressed as the concentrations capable of inhibiting fifty percent of the oxidation of DHR (IC_{50}).

Trolox equivalent antioxidant capacity assay (TEAC)

To measure the antioxidant capacity, the TEAC assay was widely used (27, 28, 29). This assay assesses the total radical scavenging capacity of polyphenols, based on the ability of a compounds to scavenge the stable ABTS radical (ABTS $^+$) formed by adding $K_2S_2O_8$ to ABTS. This method measure the reaction of the blue/green stable ABTS radical with antioxidants. In the reaction with antioxidant polyphenols, the blue/green color disappears. This decolorization is determined spectrophotometrically at 734 nm after 30 min from adding polyphenol samples. The reduction in absorbance is related to that of Trolox, a synthetic, hydrophilic vitamin E analogue, which gives the TEAC value. The TEAC is calculated as moles of Trolox equivalents per gram of extract or polyphenol compound.

Reducing Effects

Ferric-reducing antioxidant power assay (FRAP)

The FRAP assay was carried out according to the procedure of Benzie and Strain (30) with slight modification. Briefly, the FRAP reagent was prepared in acetate buffer (pH 3.6), 10 mmol 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mmol HCl and 20 mmol iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 °C in a water bath prior to use. 15 μ l of sample were added to 1.5 ml of the FRAP reagent. The absorbance of the mixture was measured at 593 nm after 4 min. The standard curve was prepared by iron (II) sulfate solution, and the results were expressed as mmol Fe (II)/g polyphenol or polyphenol-rich extract.

Reducing power (RP)

The reducing power of phenolic samples was widely determined by the method of Jayaprakasha et al. (31). Different concentrations of polyphenols dissolved in methanol (1 ml) were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. After incubation 20 min at 50 °C, 2.5 ml of 10% trichloroacetic acid was added to the mixtures. After centrifugation, 2.5 ml of the upper layer were diluted with distilled water and 0.5 ml of 0.1% ferric chloride was added. The absorbance was measured at 700 nm. Increase in absorbance of the reaction indicated the reducing power of the polyphenol test samples.

Phosphomolybdenum method

The assay is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH. Total antioxidant capacity of polyphenol was evaluated according to the method described by Jayaprakasha et al. (32). An aliquot adequately diluted of samples (0.1 ml) was combined with 1.0 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a boiling water bath at 95 °C for 90 min. Then, the samples were cooled to room temperature and the absorbance was measured at 695 nm against blank prepared in the same conditions by replacing sample with 0.1ml of methanol. Antioxidant capacity was expressed as ascorbic

acid equivalents (mmol/g).

Inhibition of Lipid Peroxidation

Measurement of thiobarbituric acid-reactive substances (TBARS)

The level of lipid peroxidation products in tissues or plasma lipid was measured as thiobarbituric acid reactive substances (TBARS) according to the modified method of Park et al. (33). To 40 µl of plasma or tissue lipid sample were added 10 µl of CuSO₄ (0.33 mg/ml) and polyphenol solution at different concentration. The preparation was incubated 24 h at 30 °C and then 100 µl of SDS 8.1% were added. After incubation 60 min at room temperature, 250 µl of trichloroacetic acid (20%, pH 3.5) and 250 µl of thiobarbituric acid 0.8 % were added. The mixture was stirred and heated 60 min at 95 °C. After cooling, 1 ml of n-butanol was added and tubes were centrifuged at 4500 rpm/15 min after agitation. The absorbance of the organic layer was measured at 532 nm. The amount of TBARS was calculated from a calibration curve of 1, 1, 3, 3-tetramethoxypropane

Mesearment of conjugated dienes

The LDL oxidation was measured as an increase in absorption at 234 nm, reflecting lipid peroxidation associated with the production of conjugated dienes which absorb at 234 nm. Thereby, a prolongation of the lag-phase indicates the higher antioxidative potential of the substance investigated (34). Polyphenol samples were mixed at different concentrations with PBS pH 7.4, CuSO₄ 1.67 µM, LDL 25 mg protein in final volume of 1ml. The absorbance was followed spectrophotometrically at 234 nm at 37 °C.

β-carotene-linoleic acid assay

Antioxidant capacity of polyphenol and polyphenol-rich extracts can be determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides formation from linoleic acid oxidation. These substances cause a discoloration of β-carotene (35). To an emulsion of β-carotene-linoleic acid mixture were added polyphenol samples at different concentrations and the emulsion system was incubated up to 48 hours at room temperature. After this incubation period the absorbance of the mixtures was measured at 490 nm. The antioxidant effect of the polyphenol was determined by comparison to control without sample.

REFERENCES

1. F.C. Lau, B. Shukitt-Hale, J.A. Joseph. The beneficial effects of fruit polyphenols on brain aging. *Neurobiol Aging* **26** (Suppl 1):128–32 (2005).
2. M.L. Neuhouser. Dietary flavonoids and cancer risk: evidence from human population studies. *Nutr Cancer* **50**:1–7 (2004).
3. J. Vaya, S. Mahmood, A. Goldblum, M. Aviram, N. Volkova, A. Shaalan, R. Musa, S. Tamir. Inhibition of LDL oxidation by flavonoids in relation to their structure and calculated enthalpy. *Phytochemistry* **62**: 89–99 (2003).
4. P.M. Kris-Etherton, K.D. Hecker, A. Bonanome, S.M. Coval, A.E. Binkoski, K.F. Hilpert, A.E. Griel and T.D. Etherton. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am. J. Med.* **113** (Suppl. 9B): 71S–88S (2002).
5. B. Fuhrman, M. Aviram. Flavonoids protect LDL from oxidation and attenuate atherosclerosis. *Curr. Opin. Lipidol.* **12**: 41–8 (2001).
6. A.S. Pannala, C.A. Rice-Evans, B. Halliwell, S. Singh. Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. *Biochem Biophys Res Commun* **232**:164–8 (1997).

7. C.G. Heijnen, G.R. Haenen, F.A. van Acker, W.J. van der Vijgh, A. Bast Flavonoids as peroxynitrite scavengers: the role of the hydroxyl groups. *Toxicol In Vitro* **15**:3–6 (2001).
8. M.R.Santos, L. Mira. Protection by flavonoids against the peroxynitrite-mediated oxidation of dihydrorhodamine. *Free Radic Res* **38**:1011–8 (2004).
9. U. Ketsawatsakul, M. Whiteman, B. Halliwell. A reevaluation of the peroxynitrite scavenging activity of some dietary phenolics. *Biochem Biophys Res Commun* **279**:692–9 (2000).
10. H.E. Hajji, E. Nkhili, V. Tomao, O. Dangles. Interactions of quercetin with iron and copper ions: complexation and autoxidation. *Free Radic Res* **40**:303–20 (2006).
11. L. Mira, M.T. Fernandez, M. Santos, R. Rocha, M.H. Florencio, K.R. Jennings. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radic Res* **36**:1199–208 (2002).
12. M.L. Neuhouser. Dietary flavonoids and cancer risk: evidence from human population studies. *Nutr Cancer* **50**:1–7 (2004).
13. S. Mandel, T. Amit, L. Reznichenko, O. Weineb, M.B. Youdim. Green tea catechins as brain-permeable, natural iron chelators–antioxidants for the treatment of neurodegenerative disorders. *Mol Nutr Food Res* **50**:229–34 (2006).
14. H. Schroeter, C. Boyd, J.P. Spencer, R.J. Williams, E. Cadenas, C. Rice- Evans. MAPK signaling in neurodegeneration: influences of flavonoids and of nitric oxide. *Neurobiol Aging* **23**:861–80 (2002).
15. V. Zbarsky, K.P. Datla, S. Parkar, D.K. Rai, O.I. Aruoma, D.T. Dexter. Neuroprotective properties of the natural phenolic antioxidants curcumin and naringenin but not quercetin and fisetin in a 6-OHDA model of Parkinson's disease. *Free Radic Res* **39**:1119–25 (2005).
16. A. Hagerman, I. Harvey-Mueller, H.P.S. Makkar. Quantification of Tannins in Tree Foliage-A Laboratory Manual. *FAO/536 IAEA Vienna*, pp. 4–7, 537 (2000).
17. M. Jay, J.F. Gonnet, E. Wollenweber, B. Voirin, Sur l'analyse 551 qualitative des aglycones flavoniques dans une optique chimiotaxonomique. *Phytochemistry*, **14**, 1605–1612 (1975).
18. C.S. Ough, M.A. Amerine. Methods for analysis of musts and wines (2nd ed.). New York: John Wiley and Sons (1988).
19. X.P. Liao, B. Shi. Selective removal of tannins from medicinal plant extracts using a collagen fiber adsorbent. *J Sci Food Agr* **85**: 1285–91 (2005).
20. A.E. Hagerman, L. G. Butler. Protein precipitation method for the quantitative determination of tannins. *J Agr Food Chem* **26**: 809–12 (1978).
21. M. Burits, F.Bucar. Antioxidant activity of Nigella sativa essential oil. *Phytother Res* **14**: 323–28 (2000).
22. M. Cuendet, K. Hostettmann, O. Potterat. Iridoid glucosides with free radical scavenging properties from *Fagraea blumei*. *Hel Chim Acta* **80**: 1144–52 (1997).
23. C. Beauchamp, I. Fridovich. Superoxide dismutase: improved assay and an assay applicable to polyacrylamide gels. *Anal Biochem* **44**: 276–87 (1971).
24. M. Suzuki, T. Watanabe, A. Miura, E. Harashima, Y. Nakagawa, K. Tsuji. An extraction solvent optimum for analyzing polyphenol contents by Folin-Denis assay. *Nippon Shokuhin Kagaku Kaishi* **49**: 507–511 (2002).
25. W.H.Koppenol, R. Kissner and J.S. Beckman Syntheses of peroxynitrite: to go with the flow or on solid grounds? *Meth Enzymology* **269**: 296–302 (1996).
26. N.W. Kooy, J.A. Royall, H. Ischiropoulos and J.S. Beckman Peroxynitritemediated oxidation of dihydrorhodamine 123. *Free Rad Biol Med* **16**: 149–56 (1994).
27. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol Med* **26**:1231–37 (1998).
28. R. Van den Berg, G.R.M.M. Haenen, H. van den Berg and A. Bast Applicability of an improved TEAC assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem* **66**: 511–17 (1999).
29. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad Biol Med* **26**: 1231–1237 (1999).
30. I.F. Benzie, J.J. Strain. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal Biochem* **239**: 70–76 (1996).
31. G.K. Jayaprakasha, R.P. Singh, K.K. Sakariah. Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models in vitro. *Food Chem.* **73**: 285–90 (2001).
32. G.K. Jayaprakasha, L. Jaganmohan, K.K. RaoSakariah. Antioxidant activities of flavin in different in vitro model systems. *Bioorgan Med Chem* **12**: 5141–46 (2004).
33. S.Y. Park, S.H. Bok, S.M. Jeon, Y.B. Park, S.J. Lee, T.S. Jeong, M.S. Choi. Effect of rutin and tannic acid supplements on cholesterol metabolism in rats. *Nutr Res* **22**: 283–95 (2002).
34. H. Esterbauer, G.Striegl, H. Puhl, M. Rotheneder. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. *Free Radical Res Comm* **6**: 67-75 (1989).
35. A. Dapkevicius, R. Venskutonis, T.A. Van Beek, P.H. Linssen., Antioxidant activity of extracts obtained by different isolation procedures from some aromatic herbs grown in Lithuania. *J Food Agr Sci* **77**: 140–46 (1998).