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Evaluation of the antioxidant properties and bioavailability of free and bound phenolic acids from *Trichilia emetica* Vahl.

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Abstract

Trichilia emetica Vahl. is commonly used in folk medicine of Mali for the treatment of various diseases. In this study, the content and the antioxidant activity of phenolic acids from *Trichilia emetica* root were evaluated.

Free phenolic acids were extracted with a mixture of methanol and 10% acetic acid. Bound phenolic acids were released using first alkaline and then acid hydrolysis. All fractions were quantified separately by HPLC. After alkaline hydrolysis, a remarkable increase in caffeic acid, ferulic acid, *p*-coumaric acid, syringic acid, vanillic acid, protocathecuic acid and gallic acid content was observed, showing that most of phenolic acids in the drug are present as bound forms. Moreover, the extracts submitted to alkaline hydrolysis showed high antioxidant properties in two in vitro assays: autooxidation of methyl linoleate (MeLo) and ascorbate/Fe^{2+}-mediated lipid peroxidation in rat microsomes.

An in vivo study was also performed to investigate the intestinal absorption of phenolic acids after oral administration of *Trichilia emetica* extracts. Results showed high levels of phenolic acids, free or conjugated to glucuronide, in the plasma of rats treated with the hydrolyzed extract. Due to the absence of chlorogenic acid in plasma samples, the presence of caffeic acid seems to be derived from hydrolysis of chlorogenic acid in the gastrointestinal tract.

Keywords: *Trichilia emetica*; Antioxidant activity; Phenolic acids; Lipid peroxidation; Methyl linoleate

1. Introduction

Trichilia emetica Vahl. (Meliaceae) is a tree widely distributed in Tropical Africa, commonly used in the folk medicine of Mali for the treatment of various disease, as a purgative, antiepileptic, antipyretic, antimalarial agent and in the hepatic disorders (Iwu, 1993). Previous phytochemical studies have reported the isolation of limonoids (Nakatani et al., 1981, 1985) from the root. The presence of tannins in the root and stem bark was also reported (Burkill, 1997). Our recent studies confirmed the high polyphenolic content of an aqueous extract from the root which could be responsible for the hepatoprotective effects against CCl_4-induced liver damage in vivo (Germanò et al., 2001) and in vitro (Germanò et al., 2005). Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids. In the past 10 years, they have received particular attention due to their role in the prevention of several human diseases, particularly atherosclerosis and cancer, for their potent antioxidant properties (Mattila and Kumpulainen, 2002). The most representative of hydroxycinnamic acids is caffeic acid which occurs in plants mainly in esterified form as chlorogenic acid. Hydroxycinnamic and hydroxybenzoic acid derivatives are rarely present in plants as free forms. More often, they occur as esters, glycosides and bound complexes. The bound forms are released by an alkaline or an acid hydrolysis, or both (Rommel and Wrolstad, 1993). In the present study, free (nonhydrolyzed) and bound (hydrolyzed) phenolic acids in *Trichilia emetica* root extracts were quantified. The antioxidant properties of *Trichilia emetica* extracts were also tested in vitro both on autoxidation of methyl linoleate

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(MeLo) and ascorbate/Fe^{2+}-mediated lipid peroxidation in rat liver microsomes. Moreover, because polyphenols in the form of esters, glycosides or polymers are poorly absorbed (Manach et al., 2004), an in vivo study was performed to evaluate the absorption of phenolic acids from their bound forms in plasma samples after an oral short-term administration of *Trichilia emetica* extracts.

2. Materials and methods

2.1. Chemicals

β-Glucuronidase (EC 3.2.1.31) type IX-A from *Escherichia coli*, caffeic acid, 5′-caffeoylquinic acid (chlorogenic acid), ferulic acid, *p*-coumaric acid, syringic acid, vanillic acid, protocathecuic acid, gallic acid, linoleic acid methyl ester (methyl linoleate), 2,2,4-trimethylpentane (isooctane) and thiobarbituric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA); butylated hydroxytoluene (BHT) was from Fluka (Fluka Chemie AG, Buchs, Switzerland). All organic solvents were obtained from Carlo Erba (Milano, Italy).

2.2. Plant material

Plant material (root) was collected in the belt of Bamako (Mali) and authenticated by comparison with a voucher specimen of *Trichilia emetica* Vahl. (No. 0651) deposited at the herbarium of Traditional Medicine Division of Bamako (Mali).

2.3. Hydrolysis and extraction of phenolic acids from plant material

Determination of free and total phenolic acids in the root of *Trichilia emetica* was done according to the method of Mattila and Kumpulainen (2002) modified.

Briefly, 5 g of dried root were homogenized in 70 ml of a mixture of methanol (containing 2 g/l of 2,(3)-tert-butyl-4-hydroxytoluene) and 10% acetic acid (85:15) using an Ultra-Turrax (Janke and Kunkel KG, IKA-WERK, Staufen, Breisgau) homogenizer. After homogenisation, the mixture was ultrasonicated for 30 min, then it was made up to 100 ml with distilled water, filtered and evaporated to dryness (yield 208.86 mg/g of dry drug). A part of this solid residue was used for HPLC analysis of free phenolic acids, the remaining ones was diluted with distilled water (120 ml) and then submitted to alkaline hydrolysis by adding NaOH (10 M, 50 ml) under a nitrogen flow at room temperature for 4 h. After incubation, the pH was adjusted to 2.0 with HCl (4N). The released phenolic acids were extracted with a mixture of diethyl ether (DE) and ethyl acetate (EA) (1:1) (3 × 300 ml, 30 min each). Finally, the DE/EA fractions were combined, evaporated to dryness (yield 40.68 mg/g of dry drug) and stored at −20 °C overnight before HPLC analysis.

After alkaline hydrolysis was completed, the aqueous fraction residue was submitted to an acid hydrolysis by adding 25 ml of concentrated HCl and incubating in a water bath (85 °C) for 30 min. After cooling, the pH was adjusted to 2.0. The DE/EA extraction performed was similar to that for alkaline hydrolysis. Finally, the DE/EA fractions were combined, evaporated to dryness (yield 6.8 mg/g of dry drug) and stored at −20 °C overnight before HPLC analysis.

2.4. HPLC analysis

Isocratic HPLC analysis was carried out on a Hewlett Packard liquid chromatograph (HPLC 1100) equipped with quaternary solvent pump, an autosampler, a thermostat and a photo diode array detector. A Spherisorb S50DS1 reversed-phase column (250 mm × 4.6 mm) with a ODS guard-column (4 mm × 20 mm) were used with a mobile phase consisting of 86.5% of acetic acid (2%) and 16.5% of tetrahydrofuran at a flow rate of 1 ml/min and at the temperature of 40 °C.

Phenolic acids were identified by matching the retention time and spectral characteristic against those of standards. Calibration curve of the standards revealed good linearity with R^2 value exceeding 0.999 (peak areas versus concentrations). Quantification was carried out using *m*-coumaric acid (1 mg/ml) as external standard.

2.5. Autoxidation of methyl linoleate (MeLo)

The inhibitory effects of *Trichilia emetica* on autoxidation of MeLo were evaluated with the method of Davalos et al. (2003) modified.

For the test, 10 μl of *Trichilia* extracts (nonhydrolyzed and hydrolyzed) previously dissolved in methanol, were added to 50 mg of MeLo, and vortexed for 10 s. After the methanol was evaporated under nitrogen, the mixture was placed at 40 °C in the dark. Each sample was tested in a range from 0.5 to 25 μg/50 mg of MeLo.

Sample aliquots (10 μl) were removed at times from 0 to 96 h, added to 2,2,4-trimethylpentane (990 μl) and mixed vigorously. Then, 10 μl of this mixture were diluted with 2 ml of 2,2,4-trimethylpentane for spectrophotometric measurements (Shimadzu, UV-1601) of conjugated diene absorption at 234 nm. MeLo hydroperoxides were calculated using an absorptivity of 26,000. Results were expressed as the amount of extract that inhibits hydroperoxide formation during 96 h of incubation time. The extract concentrations reducing hydroperoxide formation by 50% (IC_{50}) after 96 h were also calculated. Caffeic acid was used as reference standard. The experiments were run in triplicate.

2.6. Assay of nonenzymatic lipid peroxidation in vitro

The inhibitory effects of *Trichilia emetica* on lipid peroxidation were evaluated on liver microsomes which were isolated as described by Germanò et al. (2002). For ascorbate/Fe^{2+}-induced lipid peroxidation, the incubation mixture (1 ml) consisted of 970 μl of a microsomal suspension (previously heat-inactivated at 90 °C for 1.5 min to remove all enzymatic factors), 10 μl of *Trichilia* extracts (nonhydrolyzed and hydrolyzed, tested at various concentrations) and 10 μl of ascorbic acid (0.1 mM). The reaction was started by the addition of 10 μl of $FeSO_4$