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Study of genotoxic, antigenotoxic and antioxidant activities of the digallic acid isolated from *Pistacia lentiscus* fruits

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ABSTRACT

The digallic acid obtained from the fruit *Pistacia lentiscus* exhibits an inhibitory activity against nitrofurantoine and B[*a*]P induced genotoxicity when tested by the SOS chromotest bacterial assay system in the presence of *Escherichia coli* PQ37 strain. The antioxidant activity of the tested compound was determined by its ability to scavenge the free radical $ABTS^{+\cdot}$, to inhibit the xanthine oxidase, involved in the generation of free radicals, and to inhibit the lipid peroxidation induced by H_2O_2 in the K562 cell line. Our results revealed that digallic acid shows an important free radical scavenging activity towards the $ABTS^{+\cdot}$ radical (99%) and protection against lipid peroxidation (68%).

1. Introduction

In recent years, focus on plant research has increased all over the world. Many plant extracts have demonstrated potent cancer chemopreventive properties as observed in the last decade (Ames, 1998; Beckman and Ames, 1998). Most of these extracts are known to exert their effects via antioxidant mechanisms either by quenching reactive oxygen species (ROS), inhibiting lipid peroxidation or by stimulating cellular antioxidant defenses (Park and Pezzuto, 2002; Valko et al., 2007). Natural antioxidants that can neutralize ROS include, cysteine, reduced glutathione, polyphenolic compounds (anthocyanins, flavonoids, phenolic acids), carotenoids (α-carotene, β-carotene lycopene), ascorbic acid (vitamin C), α-tocopherol (vitamin E), and indole carbinols (Shahidi et al., 1992). Taking into consideration the several drawbacks of synthetic compounds for the human organism, the examination of plant origin preparation has received specific attention. Nowadays, about 4000 compounds of polyphenolic structures are known. They exert a wide variety of biological features including anticarcinogenic, antimutagenic and antioxidative activities (Yagi et al., 2002; Yilmaz et al., 2007; Hayder et al., 2004, 2005; Mertens-Talcott and Percival, 2005; Kilani et al., 2005; Bala et al., 1997; Ben Ammar et al., 2008).

Since many xenobiotic substances may damage DNA strands and cause lipid peroxidation (Park et al., 1996), we have attempted in this study to find new anticarcinogenic and antioxidant compounds and to determine: the radical scavenging capacity, the anti-lipid peroxidation and antigenotoxic effects of active principles isolated from the most active ethyl acetate (EtOAc) fraction of *Pistacia lentiscus* fruits, namely, digallic acid (DGA).

P. lentiscus L. is an evergreen member of the Anacardiaceae family, largely distributed in "extreme" ecosystems of Mediterranean area, which are characterized by nutrient and water scarcity and long term exposures to extensive solar radiation and high temperatures (Margaris, 1981).

2. Materials and methods

2.1. Plant materials

The fruits of *P. lentiscus* L., were collected from Zaghouane situated in the north of Tunisia, in November 2003. Identification was carried out by Pr. Chaieb (Department of Botany, Faculty of Sciences. University of Sfax), according to the flora of Tunisia (Cuénod, 1954). A voucher specimen (Pl-11-03) has been kept in our laboratory for future reference.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-sulfonic acid)diammonium salt; AP, phosphatase alcaline; B(a)P, benzo(a)pyrène; CH_2Cl_2-MeOH, dichloromethane-methanol; CML, human chronic myelogenous leukaemia; DGA, digallic acid; EtOAc, ethyl acetate; H_2O_2, dihydrogene peroxide; β, gal:beta-galactosidase; IF, induction factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MDA, malonaldehyde; X, xanthine; XOD, xanthine oxidase.

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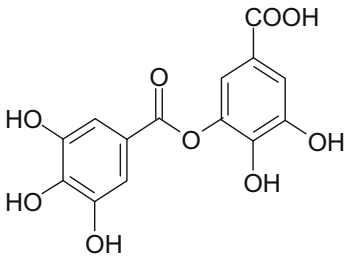

Fig. 1. Digallic acid.

2.2. Extraction method

The powdered fruits were extracted with boiling water for 15–20 min. After filtration, the extract was filtered and lyophilized leading to an aqueous one. The residue was suspended in water and successively portioned between water and chloroform, ethyl acetate and 1-butanol. Each liquid–liquid extraction was carried out three times (water:organic solvent = 1:1 v/v). The solvents of the obtained sub-extracts were evaporated under vacuum to dryness.

The ethyl acetate soluble fraction (2 g) was fractionated over silica gel column eluted with CH_2Cl_2–MeOH gradually increasing the MeOH content and three fractions were collected. Fraction 1 was rechromatographed over Sephadex LH20 eluted with 100% MeOH and nine sub-fractions were obtained. Sub-fraction 7 was further purified over Sephadex LH20 column eluted with MeOH–H_2O (9:1) and nine sub-fractions were obtained. Sub-fraction 6 was purified by passage through (C18) disposable extraction column eluted with methanol and water content to afford 24.5 mg of digallic acid (Fig. 1).

2.3. Nuclear magnetic resonance (NMR)

NMR spectroscopy experiments on the compounds were performed on a Bruker_Avance 400 at 400 MHz (for ^{1}H NMR) and 100 MHz (for ^{13}C NMR) with CD3OD as solvent. FAB–MS (negative-ion mode, glycerol matrix) was recorded on an R210C (VG Instruments, Altrincham, UK) spectrometer equipped with an IPC (P2A) MSCAN WALLIS computer system. COSY, HMQC, and HMBC spectra were obtained using the usual pulse sequences.

2.4. Cell culture

Human chronic myelogenous leukaemia (CML) cell line K562 was obtained from the American Type Culture Collection (Rockville, MD). It was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% gentamycine and 2 mM L-glutamine. All cells were incubated at 37 °C in a humidified atmosphere enriched with 5% CO_2.

2.5. Activation mixture

The S9 microsome fraction was prepared from the liver of rats treated with Aroclor 1254 (Maron and Ames, 1983). The composition of the activation mixture is the following per 10 ml of S9 mix: salt solution (1.65 M KCl + 0.4 M $MgCl_2 \cdot 6H_2O$) 0.2 ml; G6P (1 M) 0.05 ml; NADP (0.1 M) 0.15 ml; Tris buffer (0.4MpH7.4) 2.5 ml; Luria broth medium 6.1 ml; S9 fraction 1 ml.

2.6. SOS chromotest

The SOS chromotest was employed to determine the effect of digallic acid (DGA) on the genotoxicity of c (B[*a*]P: indirect acting mutagen), and indirect acting nitrofurantoine (direct acting mutagen) induced genotoxicity.

The SOS chromotest with *Escherichia coli* PQ37strain was performed according to the procedure described by Quillardet and Hofnung (1985). The genotype of this strain is: F– *thr leu his-4 pyrD thi galE galK lacΔU169 Srl300::Tn10 rpoB rpsL uvrA rfa trp::Muc + sfiA::Mud* (Ap, *lac*) *cts*. An exponential-phase culture of *E. coli* PQ37 was grown at 37 °C in LB medium to an approximate cell density of $2 \cdot 10^8$ cell/ml supplemented with ampicillin (20 μg/ml). One ml of this culture was diluted with 9 ml of fresh medium; DGA was dissolved in 1% dimethylsulfoxide (DMSO), three concentrations of this compound (1, 5, and 10 μg/assay) were prepared and tested in triplicate with and without an exogenous metabolic activation system. Positive controls was prepared by exposure of the bacteria to either B[*a*]P or nitrofurantoine. After 2 h of incubation at 37 °C, with shaking, 300 μl samples were used for assaying β-galactosidase (β-gal) and alkaline phosphatase (AP) activities. In this assay, the β-galactosidase synthesis (*lacZ* gene) is dependent on *sfiA* activation and is used to measure induction of SOS repair system. The activity of the constitutive enzyme alkaline phosphatase was used as a measure of protein synthesis and toxicity. Enzyme activities were assessed spectrophotometrically. The SOS induction factor (IF) in treated cells was obtained by comparing β-galactosidase and alkaline phosphatase activities in treated and untreated cells. The result was considered positive when the IF for β-galactosidase activity was >2.0. For evaluation of the protective effect of DGA on the induction of the SOS response by nitrofurantoine (in the absence of the S9 activation mixture) and B[*a*]P (in the presence of the S9 activation mixture), 10 μl of nitrofurantoine solution (10 μg/assay) or B[*a*]P solution (2.5 μg/assay) were added into tubes with 10 μl of the tested concentration of DGA. Antigenotoxicity was expressed as percentage inhibition of genotoxicity induced by either nitrofurantoine or B[*a*]P according to the formula:

$$(\%) = 100 - (IF_1 - IF_0/IF_2 - IF_0) \times 100,$$

where IF_1 is the induction factor in the presence of the test compound and the genotoxin, IF_2 the induction factor in the absence of the test compound and in the presence of the genotoxin, and IF_0 is the induction factor of the negative control. Data were collected as a mean ± S.D. of three experiments.

2.7. Radical-scavenging activity

An improved ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt] radical cation decolorization assay was used. It involves the direct production of the blue/green $ABTS^{+\cdot}$ chromophore through the reaction between ABTS and potassium persulfate. Addition of antioxidants to the performed radical cation reduces it to ABTS, to an extent and on a timescale depending on the antioxidant activity, the concentration of the antioxidant, and the duration of the reaction (Re et al., 1999). ABTS was dissolved in water to a final concentration of 7 mM. $ABTS^{+\cdot}$ was produced by the reaction of ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at a room temperature for 12–16 h before use. The $ABTS^{+\cdot}$ solution was diluted with ethanol to an absorbance of 0.7(±0.02) at 734 nm. In order to measure the antioxidant activity of the DGA, 10 μl of this compound at various concentrations (0.05, 0.1, 0.15, 0.2 mg/mL) were added to 990 μl of diluted $ABTS^{+\cdot}$ and the absorbance recorded every minute. The kinetic reaction stopped when the absorbance was stable. Each concentration was analysed in triplicate. The percentage decrease of absorbance at 734 nm was calculated for each point; the antioxidant capacity of the test compound was expressed in percent inhibition (%), and IC_{50} value was calculated from regression analysis.