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Original article

Antioxidant and renoprotective activities of *Ficus racemosa* Linn. stem bark: Bioactivity guided fractionation study

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ABSTRACT

Ethanolic extract of *Ficus racemosa* [FRE] was subjected to bioactivity guided fractionation by steady state and kinetic methods using nanosecond pulse radiolysis and stopped-flow spectrophotometer. HPLC fingerprinting and other antioxidant studies revealed that quantitative difference in FRE and its fractions were insignificant. Hence FRE was chosen for the evaluation of renoprotective study in mice using ischemia/reperfusion injury. Pretreatment of FRE (50 and 100 mg/kg) restored the serum biomarkers and tissue antioxidant status to near normal levels, which were altered in ischemic group. Further, HPLC studies revealed that FRE contains gallic acid (6.18 mg/g) as major constituent and it is responsible for observed beneficial effects.

1. Introduction

A majority of the present day diseases are reported to be due to the shift in the balance of the pro-oxidant and the antioxidant homeostatic phenomenon in the body. Pro-oxidant conditions dominate either due to the increased generation of the free radicals caused by excessive oxidative stress of the present day life, or the poor scavenging in the body caused by depletion of the dietary antioxidants [1,2]. Reactive oxygen species, which include superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$) are well documented as cytotoxic intermediates. These oxygen intermediates differ significantly in their interactions and can cause extensive cellular damage such as nucleic acid strand scission [3], modification of polypeptides, lipid peroxidation, etc. [4].

Ischemic reperfusion injury is an inevitable event during renal transplantation and is one of the major clinical problem causing renal cell death and acute renal failure [5]. The interruption of blood flow to the kidney causes ischemia, which is further augmented by reperfusion [6]. A complex cascade of events is involved in the pathogenesis of ischemic reperfusion injury. Activation of neutrophils, cytokines, platelets and generation of reactive oxygen species (ROS) and release of lytic enzymes are implicated as the main mechanisms of ischemic reperfusion injury, which can further lead to cell death, increased vascular permeability, tissue necrosis and multiorgan dysfunction. The endogenous antioxidants, which detoxify the free radicals, are depleted during this process [7,8]. Hence, the supply of exogenous antioxidants may be beneficial to protect the cells against such injury.

Several members of the genus *Ficus* (family: Moraceae) are being used traditionally in a wide variety of ethnomedical remedies. *Ficus racemosa* Linn., (Gular) which is one among them, is widely distributed all over India and northern Australia. All parts are medicinally important in the traditional system of medicine in India and have been used extensively for the treatment of various diseases [9]. It is reported that roots and bark are useful in the treatment of asthma, dysentery, diarrhoea and diabetes [10]. The leaf extract exhibited hepatoprotective and anti-inflammatory properties [11,12]. Chemomodulatory effect of *F. racemosa* against ferric nitrilotriaceatate (Fe-NTA) and potassium bromate ($KBrO_3$) induced renal carcinogenesis and oxidative damage response in rats was also reported recently [13,14]. However, detailed in vitro steady state and time resolved antioxidant activity of *F. racemosa* and its renoprotective ability are not yet explored. Hence, we thought it worthwhile to investigate a detailed study. We also carried out HPLC studies of ethanolic extract of *F. racemosa* (FRE) and its fractions to know the difference in chemoprofiles and to identify and quantify the major active constituents.

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2. Materials and methods

2.1. Chemicals

2,2-diphenyl-1-picrylhydrazyl [DPPH], 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) [$ABTS^{2-}$], ascorbic acid, Deoxy-D-ribose, thiobarbutyric acid [TBA], butylated hydroxyl toluene [BHT], butylated hydroxyl anisole [BHA] egg phospatidyl-choline, nicotinamide adenine dinucleotide [NADH], nitroblue tetrazolium [NBT], calf thymus DNA were purchased from Sigma chemical Co., USA. Trichloroacetic acid, trolox and phenazine methosulphate [PMS] were procured from Hi-Media Laboratories Pvt. Ltd, Mumbai, India. Mannitol was procured from SD fine chemicals, Mumbai, India. All the other chemicals used were of analytical grade, solvents for HPLC were of HPLC grade procured from Qualigens Fine chemicals, India. IOLAR grade nitrogen and N_2O were used for degassing the samples. Nanopure water from Millipore Milli-Q system was used for preparing solutions and all solutions were prepared fresh.

2.2. Plant material

The dried bark of *F. racemosa* was collected from Valsad, Gujarat, India, in the month of May 2006, identified and authenticated by a qualified botanist and a voucher specimen (#MCOPS/PC/05/06) deposited in the college herbarium.

2.3. Extraction and fractionation

The shade dried and powdered bark of *F. racemosa* (1 kg) was extracted exhaustively with 95% ethanol in a soxhlet, followed by water extraction on a hot water bath. The ethanol extract [FRE] (Herb: extract = 6.7) and water extract [FRW] (Herb: extract = 10.8) was concentrated to a small volume and then evaporated to dryness. Dried FRE (55 g) was suspended in distilled water (500 mL) and fractionated successively with the following solvents (4 × 500 mL each) to yield the following fractions: (1) petroleum ether (60–80 °C) [FRE1; yield 0.15%], (2) chloroform [FRE2; yield 7.3%], (3) ethyl acetate [FRE3; yield 23.6%] and (4) n-butanol [FRE4; yield 27.3%]. The remaining water soluble fraction was designated as FRE5 [yield 29.1%].

2.4. HPLC fingerprinting

The HPLC finger printing (LC-10AD*v*P, Shimadzu Corporation, Japan) was carried out using 700 ppm solutions of FRE, FRE3, FRE4 and FRE5 on a reverse phase packed column (RP C-18 column; Supleco, USA; 250 mm × 4.6 mm; particle size 5 μm) using gradient elution. Gradient elution was performed using water and acetonitrile at a total flow rate of 1.0 mL/min with a run time of 30 min and elution was monitored by using PDA detector; gradient composition (min, % acetonitrile): 0, 20; 5, 40; 8, 75; 12, 90; 15, 95; 25, 95; 27, 20; 30, 20. The chromatograms at 270 nm were analyzed and compared.

2.5. Estimation of bioactive-gallic acid in FRE using HPLC

For HPLC analysis, FRE was dissolved in HPLC grade MeOH (1 mg/mL) and subjected to HPLC for quantitative analysis of polyphenols. The Shimadzu (Kyoto, Japan) HPLC was equipped with dual pump LC-20AD binary system, PDA detector SPD–M20A, Merck C_{18} reversed-phase column (I.D 4.6 mm × 250 mm, 5 μ). Separation was achieved with a two-pump linear gradient program for pump A (Water containing 0.1% formic acid) and pump B (Acetonitrile). Initially run with a gradient of 10% B changing to 70% in 25 min. Flow rate and injection volume were 1.0 mL/min and 20 μL, respectively. The chromatographic peaks of the analytes were confirmed by comparing their retention time and UV spectra with those of the reference standard. Results (mg/g dry wt.) were obtained by comparison of peak areas (254 nm) of FRE with that of the gallic acid.

2.6. Free radical scavenging studies

2.6.1. Reaction with DPPH radical

The reaction of extract/fraction with DPPH was followed by steady state and time resolved methods. The steady state scavenging activity of extract and fraction (2–200 μg/mL) was measured by the method earlier described [15].

Kinetics of DPPH reaction with the extract/fractions was studied using stopped-flow kinetic spectrometer Model SX 18 MV (Applied Photophysics, UK) in single mixing mode. In this experiment, syringe I contained 50 μM DPPH in methanol and syringe II contained solutions of FRE and FRE3 to FRE5 (26–275 μg/mL) separately. With a time delay of 1.3 s, both the solutions in syringe I and II were mixed and the relative change in the absorbance at 517 nm as a function of time at 25 °C was measured. Analysis of the kinetic traces was carried out with an exponential function using built-in software. At least three independent runs were used to determine the rate constant at different concentration [16].

2.6.2. Reaction with ABTS radical anion

The reaction of extract/fraction with $ABTS^{\bullet-}$ was also followed by steady state and time resolved methods. The steady state scavenging activity of extract and fraction (2–200 μg/mL) was measured by the method described [15].

Kinetics of $ABTS^{\bullet-}$ reaction with the extract/fractions was studied using stopped-flow kinetic spectrometer in single mixing mode. In this experiment, syringe I contained 100 μM $ABTS^{\bullet-}$ in methanol and syringe II contained solutions of FRE and FRE3 to FRE5 (9.8–260 μg/mL) separately. After mixing, time-dependent absorbance changes were monitored at 645 nm. At least three independent runs were used to determine the rate constant at different concentration [17].

2.6.3. Reaction with hydroxyl radical

Steady state hydroxyl radical scavenging activity of extract/fraction (34–665 μg/mL) was measured by degradation of deoxy-D-ribose method as described [18].

Hydroxyl radical reactions were carried out using pulse radiolysis technique employing high-energy electron pulses (50 ns, 7 MeV) obtained from a linear electron accelerator and the transients detected by kinetic spectrometry. The radiation dosimetry was done using aerated aqueous solution of 0.01 M potassium thiocyanate (KSCN) [19]. The dose per pulse was 18.5 Gy. Radiolysis of water leads to the formation of three highly reactive species namely hydrogen atom ($^{\bullet}H$), hydroxyl radical ($^{\bullet}OH$) and hydrated electron (e_{aq}^{-}). The N_2O-saturated water was used to increase quantum yield of hydroxyl radicals since it quantitatively converts the hydrated electron into hydroxyl radicals. The reaction of the components of the extract/fraction was studied with these hydroxyl radicals in solution [20]. Further, competition kinetics of hydroxyl radical scavenging by extract/fractions against 250 μM KSCN at pH 7 was studied by monitoring $(SCN)_2^{\bullet-}$ absorbance at 500 nm [21].

2.6.4. Reaction with superoxide radical anion

Steady state superoxide radical anion ($O_2^{\bullet-}$) scavenging activity of extracts/fractions (23–323 μg/mL) was measured [22]. Superoxide radical anions are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT to blue diformazan. Briefly, $O_2^{\bullet-}$ were generated by adding